Scalable manipulation of proteins using magnetic adsorbents

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PH.D. THESIS

Scalable manipulation of proteins using magnetic adsorbents

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Front page images:
Left: Microtube rack with embedded neodymium magnet allowing rapid separation of magnetic support. Fabricated by Peter Meincke, Department of Systems Biology, Technical University of Denmark. *Right:* Bacitracin derivatized magnetic particles (large structures) suspended in a *Bacillus licheniformis* culture.

“Qui audet adipiscitur - Who dares wins”

Dansk resumé

Denne ph.d. afhandling beskriver et studium i anvendelsen af magnetiske adsorbentpartikler som et relevant værktøj med avancerede anvendelsesmuligheder inden for manipulationer af proteiner. Teknikken med anvendelse af magnetiske adsorbentpartikler og den tilhørende magnetiske separation går under fælles-betegnelsen High Gradient Magnetic Fishing, forkortet HGMF. Tidligere studier i HGMF har primært været rettet mod undersøgelser af anvendelsesmulighederne inden for oprensning af proteiner fra såkaldte beskidte kilder, eksempelvis celle lysater. I disse studier er det vist, at teknikken udgør et særdeles attraktivt alternativ til mere gængse oprensningsmetoder, som oftest kræver langt flere procestrin. Men teknologien rummer et endnu større potentiale inden for såkaldte nedstrømsprocesser, som udover selve oprensningen også dækker de efterfølgende trin som fører til det endelige produkt.

Fokus i denne afhandling har været at benytte eksisterende typer af magnetiske adsorbentpartikler og tilhørende magnetisk separationsteknologi som udgangspunktet for undersøgelser af teknologiens anvendelse i avancerede og komplekse processer beregnet på manipulation af proteiner. Med udgangspunkt i tre studier med stigende kompleksitet undersøges mulighederne for at anvende magnetiske partikler og den skalerbare HGMF teknologi, som metode til: (1) stabilisering af proteiner under en gæringsproces, (2) kontrol af modifikation af proteiner under en semi-kontinuerlig proces samt (3) som potentielt ”håndtag” under samling af et protein kompleks.

I det første kapitel introduceres konceptet omkring magnetiske adsorbentpartikler og HGMF, og der gives en kortfattet og relevant beskrivelse af den teknologiske status indenfor området, og dette efterfølges af baggrundsinformation knyttet til de efterfølgende forskningskapitler.

Det andet kapitel beskriver en undersøgelse af hvorledes HGMF kan benyttes til stabilisering af proteiner under en gæringsproces, og er i sin natur en forlængelse af de eksisterende demonstrerede anvendelsesmuligheder indenfor direkte oprensning. Nedbrydning af peptidbaserede produkter, som et resultat af protease aktivitet under og efter gæringsprocessen, medfører forringet kvalitet og lavere produktudbytte. Traditionelt anvendte metoder beregnet på at omgå problemet involverer tilsætning af protease inhibitorer, ændrede vækstbetingelser eller genetiske modifikationer. I dette

kapitel demonstreres et alternativ til disse- fremgangsmåder gennem anvendelse af magnetiske adsorbentpartikler og HGMF-teknologien. En gæringsproces med bakterien *Bacillus licheniformis*, hvor modelproteinet bovine serum albumin (BSA) tilsættes, viste, at magnetiske adsorbentpartikler med den kovalente bundne protease inhibitor *bacitracin* kan benyttes til direkte *in situ* indfangning af proteasen under selve gæringsprocessen. Undersøgelsen omfattede både vækstkulturer dyrket i flasker og i bioreaktorer, og viste entydigt, at behandlingen havde en kraftigt forbedrende effekt på stabiliteten af BSA. Samtidigt blev der ikke konstateret nogen negativ påvirkning af væksten, og målinger viste, at bakterien fortsatte produktionen af proteaser således, at flere behandlinger var nødvendige for at holde protease-aktiviteten på et lavt niveau.

Kapitel tre omhandler udviklingen af en semi-kontinuerlig proces til PEGylering af proteiner gennem anvendelsen af magnetiske adsorbentpartikler. PEGylering er en proces, hvorved kæder af polyethylenglycol (PEG) kovalent kobles til overfladen af proteiner. Denne proces kan ikke udføres i cellen (*in vivo*), da PEG er en syntetisk og ikke naturligt forekommende forbindelse.

PEGylering ændrer de fysisk-kemiske egenskaber af proteiner og gør dem både mere opløselige og stabile. PEG-modificerede medicinske proteiner har desuden en forbedret farmakologisk profil, herunder længere forlænget blodcirkulation og reduceret immunogenicitet. PEGylering skal foretages efter oprensningen af proteinet og gennemføres traditionelt som en portionsproces (batch) af frie proteiner i opløsning, hvilket resulterer i dannelsen af flere typer af PEG-modificerede produkter. Denne fremgangsmåde resulterer således i et blandet produkt med varierende aktivitet, som følge af et forskelligt antal koblede PEG grupper, og den efterfølgende oprensning er kompliceret og kræver mange procestrin.

I det tredje kapitel demonstreres HGMF som et potent alternativ til den traditionelle fremgangsmåde, gennem konstruktionen af en semi-kontinuerlig PEGylering proces baseret på anvendelsen af magnetiske adsorbentpartikler. Processen dækkede absorption af trypsin via benzamidine kovalent koblet til magnetiske adsorbentpartikler, den efterfølgende PEGyleringsproces og den afslutning oprensning. Det bliver demonstreret hvorledes antallet af koblede PEG grupper kan kontrolleres, og at det resulterende produkt har højere enzymatisk aktivitet sammenlignet med tilsvarende produkter frembragt på den traditionelle vis.

I det fjerde kapitel øges kompleksiteten yderligere og HGMF teknologien forsøges anvendt som platform for samling af en proteinbaseret nano-motor. Som demonstrationsmodel anvendes domænet F_1 fra ATP syntasen, som under hydrolyse af ATP roterer 360°. I dette studie blev de relevante proteiner som udgør F_1-ATPasen i bakterien *Escherichia coli* klonet, overudtrykt og oprenset, og samling af aktive komplekser demonstreret i fri opløsning. Efterfølgende blev det forsøgt at samle F_1-ATP syntasen på overfladen af en række forskellige magnetiske adsorbentpartikler, men på trods af resultater som indikerer, at det lykkedes, påvises det ikke, at disse er aktive og i stand til at hydrolyserer ATP.

I det sidste kapitel gives en generel konklusion af det beskrevne arbejde med magnetiske adsorbentpartikler og HGMF, og der angives relevante forslag til videre arbejde med teknologien.

Thesis outline

In the past, studies on the use of magnetic adsorbents for bioprocessing have mostly centered around applications for scalable, direct product capture for protein purification during downstream processing, as a substitute for chromatography with difficult to handle feedstocks. However, the magnetic attractability of the adsorbent is a unique extra property that could potentially be used for manipulating and controlling proteins during a range of new downstream and bioprocessing tasks. Downstream processing encompasses not only purification, but the steps that follow until the final product has been produced. The main focus of this study has been to employ currently available magnetic particle and separation technologies in three proof-of-concept studies to explore the potential of magnetic adsorbents as the basis for advanced and difficult downstream processing tasks. The studies build progressively in complexity and in distance from the fermenter, starting with extension of high-gradient magnetic fishing (HGMF) to a new application for fermentation broth stabilisation, to the controlled chemical modification of proteins in a semi-continuous process, and to finish with examining the potential of magnetic adsorbents for providing a platform for assembly of protein complex based nano-motors for the burgeoning nano-bio field.

The first chapter of the thesis is a brief introduction to magnetic particles for biotechnology and the concept of HGMF, and gives a brief review of the current status of the technology which is relevant to the current work. This is followed up by background information on the chemical modification of proteins by polyethylene glycol (PEGylation), and the concept of the nano-engine. The chapter finishes by defining the aims of the work and the scope of the thesis.

The first research chapter examines an extension of the use of magnetic supports from protein purification, which has been the application most studied in a bioprocess context. The new use examined for HGMF is stabilizing poly peptides during a fermentation process. Proteolytic degradation of extracellular proteins during, and even after the fermentation process, leads to decreased quality of the product and lowered yields. Traditionally the problem is overcome by addition of inhibitors, altered growth conditions or through genetic engineering. However, it is shown in this chapter that HGMF can be used directly *in situ* with the fermenter to stabilise a model protein

(bovine serum albumin) which is added to *Bacillus licheniformis* fermentations. Bactracin coupled magnetic particles are added directly to shake flask cultivations and fermentations in bioreactors and then removed by HGMF processing, after which the fermentations continue. No deleterious effects of the magnetic particles are observed, and indeed the cells continue to produce proteases. It is shown that further rounds of protease removal are required to hold the enzyme activity at a low level and reduce degradation of the model protein.

In chapter 3 the development of a semi-continuous magnetic particle-based protein PEGylation process is described. PEGylation is a synthetic, non-natural modification not possible in the cell, in which chains of polyethylene glycol are covalently attached to the surface of proteins. The procedure is used to alter properties such as solubility and stability. PEGylated proteins have been demonstrated to display improved pharmakinetic profiles, including prolonged circulation time and reduced immunogenicity and are important new drug types. PEGylation must be conducted during downstream processing after purification of the protein. Normally it is done in free solution, generating families of PEG-conjugates, which may have different levels of activity and which must be subjected to multiple rounds of further purification. In this chapter, it was shown that magnetic supports can be used conveniently, in a semi-continuous HGMF based process, to control PEGylation of the protein and to simplify removal of unreacted PEG. The process was done at small scale and benzamidine derivatized supports were used to continuously and reversibly adsorb the protease trypsin, protecting the active site. Continuous PEGylation followed immediately, and subsequently semi-continuous HGMF processing. The types of PEG conjugates produced (*i.e.* numbers of PEG groups attached) could be tightly controlled by the process and they were found to have higher enzymatic activity than those produced in free solution.

The final research chapter pushes the envelope for the use of HGMF to extreme downstream processing possibilities. Here the use of magnetic particles and HGMF to generate a process for the assembly of protein complex based nano-engines is examined. The model chosen for the demonstration is assembly of the F_1-ATPase. Normally the F-ATPase serves as the link between the proton gradient produced by metabolic processes and the formation of ATP from ADP and inorganic phosphate in the cell. The complex is divided into two district domains, namely the membrane integrated F_0 and associated

F_1. When detached from the membrane embedded F_0-part the F_1-ATPase subcomplex is capable of catalyzing the hydrolysis of ATP and has been shown to rotate, acting as a nano-engine. In this study the subunits of the F_1-ATPase were successfully cloned, overexpressed recombinantly and purified. Assembly in free solution to give active F_1-ATPase from the individual subunits was first demonstrated. Subsequently evidence for assembly of the ATPase on magnetic supports was found, although activity of the assembled nano-motor could not be confirmed.

The last chapter gives a final conclusion on the usefulness of magnetic adsorbents and HGMF for advanced downstream processing tasks and suggests future areas within the field of HGMF which should, and could, be explored to promote this exciting new approach to downstream challenges.

Preface

The work presented in this Ph.D. thesis was carried out at the Center for Microbial Biotechnology, Institute for Systems biology, Technical University of Denmark in the period from August 2005 until October 2008 under the supervision of Associate professor Timothy J. Hobley. The study is part of the STREP research project Magnetic Field Assisted Biomaterials Processing (NanoBioMag; NMP3-CT-2005-013469) funded by the European Union Sixth Framework Programme.

Being a Ph.D. student is truly a privilege and my three years at CMB has not only been an exciting professional experience, but also left me with great memories of the unique people which make this place a fantastic working environment. In fact the number of people which in one way or the other have contributed to this project, either through discussions, practical assistance or by just being a source of inspiration is too extensive to list here. However, a few people need to be specially acknowledged by name. First among these people is my supervisor Timothy Hobley who not only provided me with the opportunity to do a Ph.D study, but also took the chance of bringing me onboard an EU project, which initially was a bit outside my area of expertise. I need to thank Trine Lütken Maury, a.k.a Magnetic girl, for the excellent times we had both in the laboratory and the office, and the results we created together. Naturally, I also need to thank Torsten Lund-Olesen for the introduction to the field of microfluids and for our fruitful collaboration. Thanks should also be given to Matthias Franzreb and Jörg Becker for hosting me at the Forschungszentrum in Karlsruhe and for introducing me to Boris Kühl who needs to be thanked for an efficient and excellent crash course in the use of MALDI-TOF. Special thanks also needs to given to the "support team" Tina, Jette, Martin and Elisabeth who kept the stocks full and regardless of my weird requests for equipment, materials and chemicals always answered my calls and to Peter and Martin for the endless repairs of equipment and construction of novel gadgets. Obviously, I would also like to thank the Downstream and Bioprocessing group, past and present, for good discussions and to the members of all the "clubs" I have been part of which covered topics such as food-for-men, cake, beer and films. The Administrative staff at CMB should also be acknowledged for their support with all the paper work and for making the formalities seem simple. Finally my family and especially my son Mathias also need to get my deepest gratitude for supporting me and for putting up with my absence.

Kim Ekelund Ottow
Copenhagen, May 2009

List of poster presentations, submitted abstracts and publications

Ottow K, Petersen TL, Arnung AM, Brask J, Villadsen J and Hobley T.
Enzyme modification using Magnetic Adsorbents as "smart" handles. *International Workshop on Downstream Processing (EFB-DSP 2007), Delft, The Netherlands, 10th- 11th of May 2007.*

Ottow K, Petersen TL, Bukowska M, Käppler T, Posten C, Brask J, Villadsen J and Hobley T.
Avoiding proteolysis during fermentation by using High Gradient Magnetic Fishing. *ECB13 13th European Congress on Biotechnology, Barcelona, Spain, 16th – 19th of September 2007. Published in Journal of Biotechnology, Volume 131, Issue 2, Supplement 1, September 2007, Page S169*

Ottow K, Petersen TL, Arnung AM, Schultz N, Brask J, Villadsen J and Hobley T.
Integrated process for protein modification using smart magnetic handles. *ECB13 13th European Congress on Biotechnology, Barcelona, Spain, 16th – 19th of September 2007. Journal of Biotechnology, Volume 131, Issue 2, Supplement 1, September 2007, Page S190*

Ottow K, Petersen TL, Bukowska M, Käppler T, Posten C, Brask J, Villadsen J and Hobley T.
In-situ removal of proteolytic activity using High-gradient Magnetic Fishing
7th European Symposium on Biochemical Engineering Science, Faro, Portugal, September 7 – 10, 2008

Peer reviewed paper

Käppler T, Cerff M, Ottow K, Hobley T, Posten C, (2009)
In situ magnetic separation for extracellular protein production.
Biotechnol Bioeng. 2009 Feb 1;102(2):535-45.

Abbreviations

BAPNA	*Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride*
BCA	*Bicinchoninic Acid*
BSA	*Bovine Serum Albumin*
Da	*Dalton*
EC	*Enzyme Commission*
HAT	*Histidine Affinity Tag*
HGMF	*High Gradient Magnetic Fishing*
HGMS	*High Gradient Magnetic Separation*
IDA	*Iminodiacetic Acid*
IMAC	*Immobilized Metal ion Affinity Chromatography*
MALDI-TOF	*Matrix Assisted Laser Desorption Ionization Time Of Flight*
MBP	*Maltose Binding Protein*
ß-Me	*2-Mercaptoethanol*
mPEG	*monomethoxy Polyethylene Glycol*
mPGA	*magnetic Poly Glutaraldehyde beads*
mPVA	*magnetic Polyvinyl Alcohol beads*
PDB	*Protein Data Bank*
PEG	*Polyethylene Glycol*
SA	*Streptavidin*
SC-mPEG	*Succimidyl carbonate monomethoxy PolyEthylene Glycol*
SHM	*Staggered Herringbone Mixer*
TCA	*Trichloro acetic acid*
Tris	*Tris(hydroxymethyl)aminomethane*

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Chapter 1

Introduction

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1. Introduction

1.1 High Gradient Magnetic Fishing

High Gradient Magnetic Fishing (HGMF) is an integrated process which uses functionalized non-porous micron-sized superparamagnetic adsorbents to recover macromolecules directly from biological feed stocks. Since the introduction of the term by Hubbuch et al. (2001) who demonstrated the direct recovery of Savinase from cell free fermentation broth, several other reports have been published describing the potential of HGMF as a powerful and highly versatile generic downstream processing tool. These include recovery of solubilised inclusion bodies from disrupted bacterial cells, enzymes from cheese whey and porcine pancreatin, antibodies from blood plasma and Lectins contained in plant extracts (Gomes, 2006; Heebøll-Nielsen et al., 2003; 2004a; 2004b; Hubbuch and Thomas, 2002, Meyer et al., 2004).

The unique property of HGMF is the ability to process crude colloidal liquids and this is due to the magnetic properties of the adsorbents used, which after loading, can be selectively removed from suspensions using High Gradient Magnetic Separation (HGMS) (Hubbuch, 2000). During traditional downstream processing of bio feedstocks it is usually necessary to perform one or more pre-treatment steps (e.g., filtration, centrifugation, precipitation) to remove suspended particles or colloidal materials prior to purification using conventional packed bed chromatography. However, as this is not required for HGMF, several unit operations may be circumvented thus reducing costs and loss of product (Franzreb et al., 2006; Hubbuch, 2000; Hubbuch and Thomas, 2002). In addition to the original purpose of HGMF as a purification tool, the potential of the method for other processes has begun to be realised and over the last couple of years. It has been demonstrated as an excellent method of controlling tryptic hydrolysis of proteins and even for continuous protein refolding and capture (Ferre, 2005; Gomes, 2006; Petersen, 2007). In the area bordering HGMF, magnetic particles combined with HGMS have also been demonstrated as a means of sorting cells and cell organelles, and as carriers of irreversibly immobilized enzymes for repeated recycling (Safarík I & Safaríková M., 1999; Schultz el al. 2007).

1.1.1 The basic principle of High-Gradient Magnetic Fishing

A magnetic fishing process consists of two simple steps which involve addition and adsorption (part I) followed by separation, washing and elution (part II) (figure 1.1). Adsorption of the protein target to the added adsorbents usually occurs relatively fast (within minutes) as the magnetic particles are non-porous. It is carried out under conditions which favour binding (e.g. pH, temperature, salt types and concentration etc.). After successfully performing the first part of the process, the next step is to separate and recover the support (and thus the adsorbed protein) from the suspension. This may be achieved using a simple bar magnetic (figure 1.1) or a HGMS system. Desorption requires that the conditions are changed from those promoting binding to those favouring release of the bound targets. Much like traditional chromatography this may be achieved by altering physiochemical parameters or by introducing a substance which binds competitively to the support (Franzreb et al., 2006; Amersham Bioscience, 2001). Normally, and much like standard chromatography, one or more washing steps may also be required to remove non-specifically adsorbed materials prior to the elution step. If a high-gradient magnetic separator is used to handle the magnetic supports during the whole process, it is termed high-gradient magnetic fishing. Use of HGMS provides a way of scaling up the handling of the magnetic adsorbents.

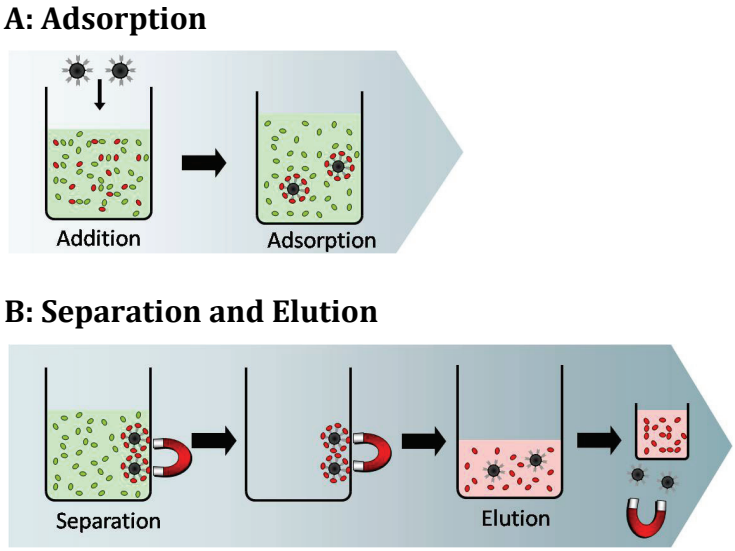


Figure 1.1: Principle steps of Magnetic Fishing. Two serial steps are employed: **(A)** Non porous magnetic adsorbents are added to the solution containing the protein of interest and incubated to achieve adsorption. **(B)** Following adsorption a step of magnetic separation is performed and in the simplest case a final step of elution.

When combined with a recycle loop, it is possible to wash, elute and regenerate the magnetic adsorbents *in situ* (see figure 1.2). Traditionally the high gradient magnetic separator is composed of a wire filter which distorts the magnetic field generating high magnetic field gradients, which improve capture of the adsorbents (for a recent review see Franzreb et al., 2007).

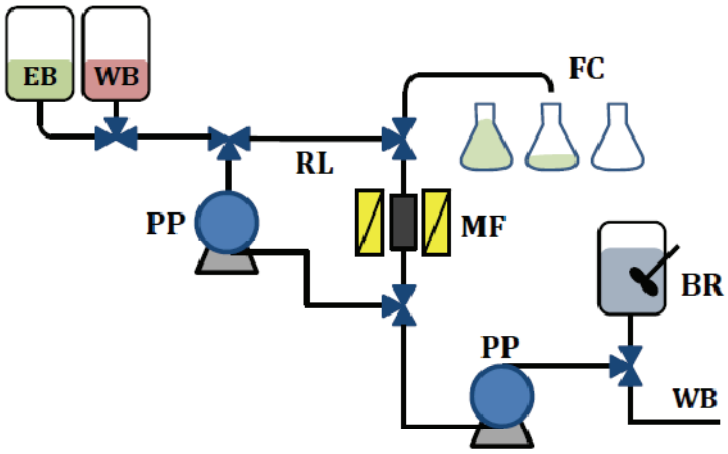


Figure 1.2: Schematic of a typical high-gradient magnetic separation system. *MF:* Magnetic filter, *FC:* Fraction Collector, *PP:* Peristaltic Pump, *WB:* Washing Buffer, *EB:* Elution Buffer container, *RL:* Recycle Loop and *BR:* Batch adsorption Reactor. Adapated from Gomes (2006)

The adsorption of protein by HGMF supports is a dynamic equilibrium in which the target protein (P), together with ligands (L) coupled to the support, forms a complex (PL) governed by an equilibrium constant (k) (equation 1.1, 1.2)

$$[P] + [L] \rightleftarrows [PL] \tag{1.1}$$

$$k = \frac{[PL]}{[P] + [L]} \tag{1.2}$$

However, the sorption equilibria between the target protein and the ligands linked to the support is typically described by the Langmuir model (Langmuir, 1918), which was originally derived to describe the adsorption of gas molecules to a plane metal surface. The model assumes that the target molecule and the ligand only have a single point of interaction (binding site), however this assumption is rarely fulfilled in the case of proteins, and adsorbents as these types of system are somewhat more complicated as compared to the original ones investigated by Langmuir. Nevertheless, the Langmuir

model (equation 1.3) still provides a practical and fairly reasonable approach to quantitatively determining the equilibrium state of adsorption by HGMF-type supports (Franzreb et al., 2007).

$$Q^* = Q_{Max} \cdot \frac{C^*}{C^* + k_d} \tag{1.3}$$

Q* describes the equilibrium loading of protein on the magnetic supports, C* refers to the amount of the target protein found in the supernatant after binding and Q_{Max} is the maximal theoretical loading capacity of the support. The dissociation constant (k_d) describes the concentration of the unbound target protein in the supernatant at which half the binding sites on the support are occupied, or in mathematical terms: Q_{kd} = ½·Q_{Max}. However, k_d also describes the stability of the complex formed between the target protein and the ligand Thus the lower the k_d, the steeper the batch binding isotherm, the more stable the complex and the greater the affinity. One of the most important and critical points in the binding step is the effectiveness of adsorption. This parameter is described by the initial slope of the binding isotherm, which may be determined by dividing the maximum binding capacity with the dissociation constant (Q_{Max}/k_d). The Langmuir parameters (Q_{Max}, k_d, Q_{max}/k_d) are naturally dependent on the protein/ligand system in question, but k_d should be in the range of 10^{-6} – 10^{-8} M to ensure efficient capture and also permit subsequent release of the bound material during elution. The maximum binding capacity (Q_{Max}) and tightness-of-binding Q_{Max}/k_d should preferably be larger than 100 mg/g support and 5 l/g, respectively (Franzreb et al. 2007).

1.1.2 The use of High Gradient Magnetic Fishing for other tasks

As mentioned briefly above, the use of HGMF is not only limited to primary recovery during downstream processing and a small number of other applications have been investigated in the literature. One of these includes controlling the hydrolysis of whey with added trypsin, by using HGMF to specifically remove the enzyme when the desired degree of hydrolysis had been reached (Gomes, 2006). The continuous capture of

refolded major histocompatibility complex (MHC) class I protein complexes has also been reported (Ferre, 2005). Furthermore, the PEGylation of proteins temporarily immobilised on magnetic adsorbents was investigated by Petersen (2007) in batch reactions. The ability to swiftly replace buffers, reagents and carry out washing steps without the loss of bound product, makes magnetic supports and HGMF an exciting new technology. It could potentially serve to overcome many problems encountered using more traditional unit operations, such as batch reactions in free solution, or on solid supports in packed beds, or on dispersed conventional solid supports that must be recovered by centrifugation. One example of this could be modification of proteins which, if bound to a non-porous magnetic adsorbent, could be conducted within a very short time span and then immediately removed from the reaction. This would also serve to allow direct recovery of the modified target protein by release from the support as a pure product without the need for additional treatment.

With the exception of the study by Ferré (2005), all investigations using magnetic supports and HGMF have been conducted as batch-wise procedures in which a defined volume was processed by the HGMF system in a stepwise manner. This includes the so-called 'semi continuous' processes of e.g. Gomes (2006) in which the adsorption steps were conducted batch-wise. This is despite the finding by Ferré (2005) that proteins can be bound to magnetic particles in continuous pipe reactors with defined residence times for unlimited time (in theory). The main reason for the lack of a truly continuous magnetic particle based bioprocess has been the limitations of the classical approach to magnetic particle separation, which has been based on the use of HGMS using filter canisters, which have limited capacities. However, as HGMS technology is also being developed, new approaches to the capture of magnetic supports are emerging. One very promising example of this is described by Stolarski et al. (2008), which uses a centrifugal approach to the challenge of magnetic separation, which allows continuous processing and theoretically unlimited capacities. Given the promise of truly continuous magnetic particle handling, it is thus timely to investigate new applications of the supports, which can solve current bioprocess and downstream processing problems as well as to look at those likely to emerge in conjunction with new fields of endeavour such as nano-biotech and synthetic biology.

1.2 Avoiding proteolytic degradation in fermentations

Expression of recombinant proteins is not a trivial matter and in order to succeed several issues have to be carefully considered. Commonly confronted questions such as the choice of expression system (e.g., bacteria or eukaryotic cells), full length or fragmented code, codon usage and many others have to addressed, in order to achieve a satisfactory result (Gräslund et al., 2008). However, from a downstream perspective, if the ultimate goal is to easily produce large amounts of protein on an industrial scale a host system which ensures that the product is efficiently secreted to the medium is to be preferred. From the perspective of product yield, bacterial systems present the best choice, however a common problem encountered in these systems is the presence of extracellular proteolytic activity originating from the organism. Examples in the literature describing the problem include α-amylase production by *Bacillus subtilis* (Nurmatov et al. 2001), lipase production by *Acinetobacter calcoaceticus* (Kok et al., 1996) and recombinant human growth hormone by *Bacillus brevis* (Kejino et al., 1997). It may thus be beneficial to remove troublesome proteases directly from the fermentation broth during the cultivation. Given that the recovery of magnetic supports directly from crude liquors has been documented in many studies, HGMF based processes may be especially suited to this task.

In a recent review (Franzreb et al., 2007), one of the major problems discussed with the use of magnetic adsorbents for direct capture of products was the lack of sufficient protein binding capacity of the current state of the art (being a function of both the adsorbents characteristics and the HGMF systems). However, it is to be expected that only trace amounts of proteases cause instability of a product during fermentation and which need to be removed. This application may thus be especially suited to the current generation of HGMF processes. Nevertheless, few previous works have reported the use of magnetic supports in fermenters (Ottow et al., 2008; Käppler et al., 2009) and it is still unclear how the fermentation kinetics are affected. Furthermore, given that fermentations must be conducted aseptically, an effective means of sterilising the magnetic supports without damaging the adsorbent is required.

1.3 PEGylation

The phrase PEGylation is used to describe a process by which polyethylene glycol (PEG) is covalently attached to the proteins and has become one of the most successful methods by which properties like solubility, stability and even immunogenicity may be improved. The term PEGylation was originally defined in the 1970's as a process by which chains of polyethylene glycol are covalently attached to drug molecules, however today the term PEGylation has developed into a more narrow definition, which more specifically deals with the process of modification of poly peptide based drugs as well as enzymes for more technical applications (Hamidi et al., 2006; Kodera et al., 1998).

Polyethylene glycol is, as the name may suggest, a synthetic polymer belonging to the polyol class, and may be found in a simple linear form as well as in branched modes in sizes ranging from a few hundred to more than 50,000 Dalton. PEG is amphipathic and PEGylated poly peptides display improved solubility in aqueous conditions as well as in some organic solvents.These properties are believed to be caused by the combined result of water binding and backbone flexibility, which makes the PEG molecule behave as if it was 5-10 times larger than a protein of similar molecular size (i.e. hydrodynamic radii) (Desantis and Jones, 1999, Fee and Van Alstine, 2005, Inada et al., 1984, Ljunger et al., 1993, Roberts et al., 2002).

When administrated intravenously, PEG below 400 Da is degraded by alcohol dehydrogenase into toxic metabolites, however in sizes above 1000 Da, PEG is considered safe. For example as much as 16 g per kg weight of a 10% solution of 4000 Da PEG can be safely administrated intravenously to rats, guinea pigs and monkeys, and has therefore been approved by the U.S. Food and Drug Administration (FDA) for internal consumption. For sizes below 20 kDa, PEG is cleared from the blood without structural changes by the kidneys and excreted in the urine, while sizes above that are only slowly removed in both urine and feces (Roberts el al., 2002, Kodera et al., 1998).

Even more interesting to the pharmaceutical industry is the observed improved pharmakinetic properties of PEGylated proteins, including extended life span in the circulation and reduced drug elimination. It is believed that the improved properties result from the increase in molecular size of the drug, which causes slower systemic clearance and protection from proteolytic degradation and enzymatic modification

caused by steric hindrance. Furthermore highly PEGylated drugs may initially be administered in an inactivate state and gradually become active as PEG groups are hydrolysed off the compound, resulting in a controlled drug release and a prolonged drug profile (Hamidi et al., 2006).

PEG is only weakly immunogenic even at high molecular weights, and even though anti-PEG antibodies have been raised, there are no known situations where this has been achieved under normal conditions. The shielding effect of PEG polymers attached to the surface of a potential antigen prevents the immune system from getting in direct contact and thus to activate an immune response (Abuchowski et al., 1977; Roberts et al., 2002; Sehon and Lee, 1977).

The general formula of PEG is HO-$(CH_2CH_2O)_n$-OH, and the PEG polymer is synthesized by an anionic ring opening polymerization reaction of ethylene oxide by nucleophilic attack of hydroxide ion on the epoxide ring. In order to couple PEG to the target molecule it is necessary to activate the polymer. This is achieved by preparing PEG derivatives with one or more reactive functional groups, and the choice is governed by the type of chemistry suitable for use with the intended target. However since one is dealing with labile molecules, in all cases the chemistry needs to be mild. In most cases the choice of PEG for protein modifications is monomethoxy polyethylene glycol (i.e. mPEG), which has one terminal hydroxy-group exchanged with a methoxy–group, which avoids cross-linking of PEG chains during their attachment to proteins. The general structure of mPEG is CH_3O-$(CH_2CH_2O)_n$-OH, and in this case the reaction is initiated by methoxyl-ions (Roberts et al., 2002). Compared to other polymers, PEG can be synthesized in a fairly narrow size distribution. However PEG is still polydispersed, with M_w/M_N ratios from 1.01 for low molecular weight polymers up to around a few thousand Da, to 1.1-1.2 for sizes above 20,000 Dalton (Roberts et al., 2002; Veronese 2001). An example of the polydispersity of activated PEG measured by MALDI TOF is shown in figure 1.3A.

PEG groups can be attached to poly peptides through several amino acid residues including serine, threonine, tyrosine, cysteine, histidine, arginine, aspartic acid and glutamic acid, however the most common choice is via the ε-amine found in the lysine side chain and the α-amine found in the n-terminal (Fee and Van Alstine, 2005). However, since lysine is a very abundant residue accounting for as much as 10% of the

primary sequence, any random PEGylation reaction will result in a heterogeneous mixture of isomers. The number of possible positional isomers (P) may be calculated using the following formula: $P = N!*((N-k)!*k!)^{-1}$, where (N) represents the possible sites and (k) the number of actual modified residues (Roberts et al., 2002).

The two most commonly used first generation PEGylation chemistries were aimed at primary amines and are succinimidyl carbonate (SC-PEG) (figure 1.3B) and benzotriazole carbonate (BTC-PEG), which both form carbamate bonds between the PEG molecule and the poly peptide. Under slightly acidic conditions histidine and tyrosine may also be targeted. Both SC-PEG and BTC-PEG are subject to hydrolysis in aqueous solution, and at pH 8 and 25°C the half-life is approximate 20 minutes for SC-PEG and 14 minutes for BTC-PEG (Roberts et al., 2002). To improve the performance of the PEGylation process, novel, so called "second generation" chemistries have been developed. One of the first to be introduced was mPEG-propionaldehyde, which is simpler to prepare, easier to use and substantially more stable as compared to first generation reagents (Harris and Herati, 1993). Additionally, studies have shown that the chemistry of mPEG-propionaldehyde is more selective, primarily reacting with α-amino groups (the N-terminal) due to the lower pK_a value of other nucleophiles (Kinstler et al., 1996).

1.3.1 Controlling PEGylation

One of the key challenges connected with the PEGylation process is controlling the number of positional isomers and retaining activity of the processed conjugates. The conventional approach to PEGylation is based on a batch-type procedure using freely suspended protein. This approach provides an efficient process; however a major drawback is the inability to control the reaction, which leads to families of products with too few or to many attached PEG groups. In addition to this, a common phenomena is loss of activity of the conjugates formed due to PEGylation in and around the active site, as well as subsequent problems during separation of conjugates from unPEGylated proteins and excess unreacted PEG.

Work aimed at solving the separation issues has been published using various types of traditional chromatography including size exclusion, ion exchange and hydrophobic

interaction chrotmatography (Rito-Palomares et al., 2007; Ziegemeier et al., 2008) as well as ultra-filtration (Kwon et al. 2008; Molek and Zydney, 2006). Although promising results were found, the use of these methods still needs further improvement in order to be fully optimized (Fee and Van Alstine, 2006).

Interestingly, Caliceli et al. (1993) demonstrated that reversible binding of trypsin to a large, porous chromatography type resin through interactions via the active site with the ligand benzamidine could protect the activity of the enzyme during a PEGylation procedure. Studies of PEGylation of human serum albumin and staphylokinase adsorbed to solid-phase anion exchangers have also been reported and improved performance was found as compared to PEGylation reactions conducted on freely suspended proteins (Suo et al., 2009).

The idea of using a magnetic support based approach for PEGylation was investigated in a proof-of-concept study by Petersen (2007) in which two model systems were evaluated in batch-wise reactions. While the study of PEGylation of savinase reversibly immobilized on bacitracin derivatized magnetic supports was unsuccessful, a completely different result was obtained for the study of trypsin bound to benzamidine linked magnetic particles. The results clearly demonstrated that the formation of PEG-conjugates could be controlled in batch reactions by manipulating the protein on the magnetic support with a bar magnet. It was found that enzyme activity could be protected and it was also shown that the removal of excess unreacted PEG was easily carried out, due to the unique magnetic properties of the support. The results presented by Petersen (2007) can serve as the starting point for further work to develop a semi-continuous and scalable PEGylation process. The SC-mPEG used in this study had an average molecular size of 2 kDa, and a MALDI-TOF spectrum showing the mass distribution is presented in figure 1.3A.

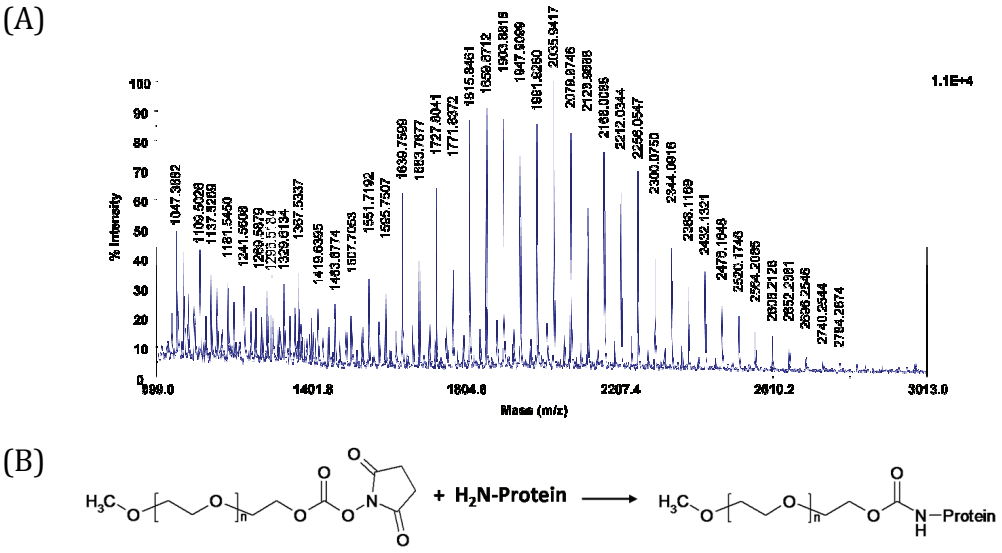


Figure 1.3: Succimidyl carbonate monomethoxy polyethylene glycol. (A) MALDI-TOF spectrum of a mono-dispersed 2 kDa SC-mPEG used in this study. **(B)** The coupling chemistry of SC-mPEG reacting with α- or ε-amino group in proteins. The succimidyl derivative was initially introduced as a PEGylation reagent by Zalipsky et al. (1992).

1.4 The concept of a nano-engine

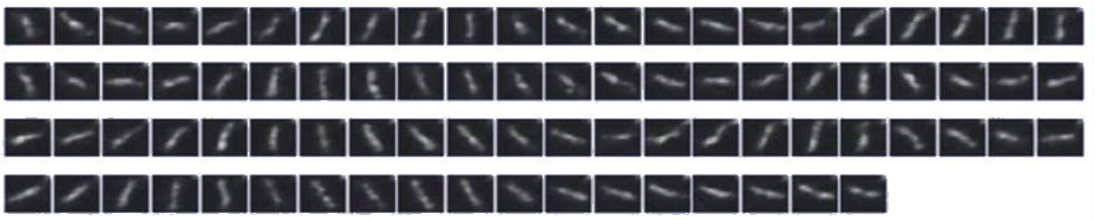
Moving structures like those found in joints are widely distributed in nature, however the concept of true continuous 360° rotation is a rare phenomenon and so far only found in connection with the flagella motor, motile cilia and the F_0F_1-ATP synthase also known as the F-ATPase. Flagella and cilia belong to a group of extracellular projections known as undulipodium or 9+2 organelles and are only associated with motile bacteria and highly specialized eukaryotic cells. The F_1-ATPase on the other hand, is found everywhere in nature and plays a key role in the production of ATP driven by energy harvested from the chemical gradient created by oxidative phosphorylation (Kinosita et al., 1998). Since the discovery of ATP by the German chemist Karl Lohnmann in 1929, and the work carried out by Herman Kalckar (Kalckar, 1937) who established the link between cell respiration and the ATP synthase, the complex has been the focus of numerous studies. Today, high resolution structural information along with biochemical and mechanical studies has made it possible to gain great insight into the mechanism behind this intriguing complex.

Structurally the ATP synthase complex consists of 20 or more individual subunits, depending on the organism, and is traditionally divided into two highly distinct sub-domains designated F_0 and F_1 (Pedersen et al., 2000). When deattached from the F_0 membrane-embedded subdomain, the membrane peripheral F_1 subcomplex can function as a rotary molecular motor driven by the hydrolysis of ATP. A direct observation of the rotation of the F_1-ATPase was published in 1997 by Noji and co-workers, who demonstrated that the central γ-subunit rotates against the surrounding αβ structure at an average rate of 1.3 revolutions per second resulting in torque equal to 40 pN·nm (Noji et al., 1997). Later studies revealed that the conversion of the energy stored in ATP into mechanical rotation is achieved with an almost 100% efficiency (Kinosita et al., 2000) and compared to an internal combustion engine, which typically offers a 20% energy conversion[1], the performance is truly quite remarkable. In addition to this, the torque-to-mass ratio of a standard car engine, which is typically around 0.8 $Nm \cdot kg^{-1}$ (based on a Mazda2 petrol internal combustion engine: 1349 cc/121 nm/151 kg) is approximately 85 times lower, as compared to that of the F_1-ATPase (68.8 $Nm \cdot kg^{-}$

[1] The efficiency is based on the information available on http://www.fueleconomy.gov.

[1]). This and other published reports on the mechanical performance of the F-type ATP synthase, as well as the growing field of nano-technology, has sparked the idea of a nano-engine, which in the near future could be used as a part of a nano-machine (Yoshida et al., 2001) (figure 1.4). When the burgeoning fields of nano-biotechnology and synthetic biology start to gain momentum, it is to be expected that efficient and scalable processes for the manufacture of large amounts of complex bio-based machines will be necessary. A magnetic, non-porous solid support may provide a convenient scaffold to assemble such bio-based machines. The non-porous nature would eliminate problems of diffusion limitations to the assembling complex, and simplify release when construction was finished, particularly if it was large and cumbersome. The magnetic properties of the support would allow efficient and controllable manipulations of the complex, with different reagents and building blocks, and the HGMF system permits a scalable and potentially continuous manufacturing process. Extensive studies of *in vitro* reconstruction of the F_1 ATPase in laboratory scale from isolated single subunits have been published as far back as 1977 (Futai et al., 1977), which make this an ideal model system for examining the potential of a HGMF based nano-engine assembly process.

(A)



(B)

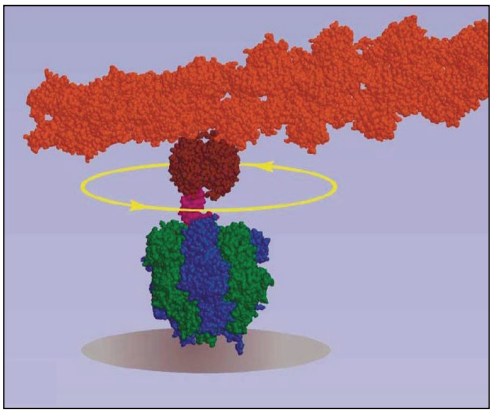


Figure 1.4: The rotation of the F_1-ATPase. (A) Sequential image analysis of the rotation. The images were obtained at 33 ms intervals and the size of the actin filament which can be seen is approximately 5 μm. **(B) The experimental system used for the observation of the rotation of the F_1-ATPase.** The space-filling model of the F_1-ATPase carrying a streptavidin attached actin filament labeled with a florescent probe (blue: α, green: β, magenta: γ, brown: streptavidin, orange: actin filament). The figure is reproduced from Yoshida et al. (2000) and includes the time lapse study originally published by Noji at al. 1997.

1.5 The aims and scope of this thesis

The broad aim of the work presented in this thesis is to expand the application of High Gradient Magnetic Fishing in the field of bio-processing and advanced downstream processing. Furthermore, to demonstrate the potency of magnetic adsorbents as a versatile and generic means for the manipulation of proteins. This is to be done using three different studies of increasing complexity, each exploring the potential of the technology in different applications, namely:

1. ***For removal of trace amounts of troublesome proteases directly during a fermentation as the means of stabilizing a protein product of interest***
2. ***As a smart handle for the scalable, continuous, controlled modification of a protein***
3. ***As the scaffold for assembly of a biological complex***

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Chapter 2

Use of High-Gradient Magnetic Fishing for Reducing Proteolysis During Fermentation

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2. Use of High-Gradient Magnetic Fishing for Reducing Proteolysis During Fermentation

2.1 Introduction

Degradation or modification of products during fermentation and downstream processing is a problem that is often caused by the side activities of extracellular enzyme by-products. One of the most common problems is the proteolytic cleavage of a protein, which complicates both laboratory and industrial scale production from a variety of hosts and numerous examples of this abound in the literature. Bock et al (2006) found that a human serum albumin-glucagon like peptide 1 fusion protein (HSA-GLP1) suffered from undesired proteolytic degradation when expressed from their usual platform process. These workers needed to develop a new downstream process specifically to reduce protease activity beyond detectable levels for production of clinical material. One of the conclusions of Wang et al. (2005) in their review of heterologous protein production by filamentous fungi is that despite achievements using molecular tools, protein loss caused by extracellular protease degradation persists. Zhao et al. (2008) reported that in 30 L pilot scale fermentations of *Pichia pastoris* the human serum albumin-interferon-alpha 2B fusion protein levels in the broth fell due to proteolytic degradation resulting from partial cell lysis. Poulsen and Petersen (1989) found that the production of amylase and cellulolytic enzymes by *Cellulomonas sp*. decreased with increasing protease formation, which was concluded to pose a serious limitation on the efficiency of the *Cellulomonas* enzyme production system. During the production of extracellular lipase (LipA) by *Acinetobacter calcoaceticus*, an extracellular serine protease was found to be responsible for degradation of the lipase desired (Kok *et al*., 1996). Kichakova *et al*. (1998) used *B. licheniformis* for α-amylase production but found that during purification there was a significant loss of amylase activity due to proteolysis, which could be partially prevented using bacitracin affinity chromatography. *Bacillus* bacteria probably represent one of the most prolific enzyme producers and would thus be an excellent choice for heterologous protein production, were it not for the proteases they naturally secrete.

To avoid proteolytic degradation of a product, different strategies can be implemented, e.g. construction of protease deficient strains (Idris et al., 2006; Kimura et al., 2008), direct inactivation of the proteases present in the fermentation broth or modification of the cultivation conditions to reduce protease formation (Zhao et al., 2008). However, none of these strategies are without drawbacks. The construction of strains deficient in unwanted proteases can be cumbersome and time consuming. Furthermore the deletion of certain proteases can result in reduced product formation or even be detrimental for the growth of the organism (Teichert *et al.*, 1989). Expression of the protein of interest in organisms which do not also have some extracellular proteases may not be possible and for certain industries the use of GMO's is simply not permitted. The standard practice of using chemically based protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) when using *Escherichia coli* for protein expression in the lab is not acceptable or possible at large scales due to toxicity. Peptide based inhibitors are expensive and not very stable in fermentation and may also be poisonous and must therefore be excluded from many applications. Modifying the fermentation conditions, such as changing temperature and pH has, in some cases, proven useful in eliminating extracellular proteolysis thereby increasing product activity (Jahic *et al*., 2003). However, restricted growth and reduced product formation can be a consequence of such changes in the process conditions.

An alternative approach for stabilising a product would be to remove the proteases directly from the broth during the cultivation using High Gradient Magnetic Fishing (HGMF), and we briefly introduced this recently (Ottow et al., 2007) as well as the use of HGMF for *in situ* downstream processing (Käppler et al., 2009). Sterile magnetic adsorbents coated with a protease specific ligand could be added during a fermentation leading to binding of the proteases present. By pumping the fermentation broth through a magnetic filter which captures the adsorbents, the unwanted trace amounts of bound proteases are removed from the bioreactor. The fermentation broth with cells and product would pass straight through the filter and go back into the bioreactor allowing formation of the heterologous protein product to continue. If more undesired proteases were produced as the cultivation continues, the cycle can be repeated until the fermentation was finished. In this paper we examine how a HGMF process employing

magnetic adsorbents can be used to remove trace amounts of proteases, which cause the unwanted degradation of target proteins in the fermentation broth. For this proof of concept study we have chosen to spike a model protein (bovine serum albumin) into *B. licheniformis* fermentations and show how the HGMF procedure can reduce degradation of the endogenous added protein.

2.2 Materials and methods

2.2.1 Materials

Bacillus licheniformis DSM 8785 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The enzymes Savinase 16L, Type EX and a purified Savinase standard were kindly donated by Novozymes A/S (Bagsværd, Denmark). Subtilisin from *B.licheniformis* (CAS 9014-01-1) was obtained from Sigma Aldrich (St. Louis, Mo, USA). All other chemicals were of analytical grade and purchased from Sigma, except where stated otherwise.

2.2.2 Manufacture and characterisation of magnetic beads

The base matrix of the magnetic adsorbents consisted of a magnetite (Fe_3O_4) core coated with aminosilane and was produced using the procedure described by Hubbuch et al. (2002). The amine-terminated supports were then coated with polyglutaraldehyde according to the procedure of O'Brien et al. (1996). Activation of the magnetic supports with divinylsulfone followed, and then coupling of bacitracin was done based on standard procedures by Hermanson et al. (1992). For initial testing, a 100 mg batch of adsorbents was made. The procedures were subsequently scaled up to produce 9 batches of adsorbents of 4 g each, which were tested and then pooled to give one 36 g batch for use in the fermenter.

The characteristics of the magnetic adsorbents were examined by constructing adsorption isotherms at room temperature using small-scale batch binding studies. In initial studies, Savinase 16L, Type EX was employed. Aliquots of ~ 2 mg of beads were equilibrated two times in equilibration buffer (100 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.5) in 2 ml tubes and magnetically separated using a 0.7 T neodymium bar magnet, after which the supernatant was removed. Solutions of Savinase in equilibration buffer were prepared and 1 mL of the protease was added to the magnetic adsorbents to give an enzyme concentration of 0-171 NPU(S)/ml (Novo Protease Units). Adsorption was then carried out for 0.5 h with shaking at ~1000 rpm on an IKA-VIBRAX-VXR shaker (IKA Werke GmbH & Co. KG, Germany). The adsorbents were then magnetically separated and the supernatant, as well as the added protease solutions, were analysed for Savinase

activity and total protein content. The exact amount of magnetic adsorbents used was determined by dry weight measurements.

The binding characteristics of the magnetic adsorbents were also examined in fermentation medium. This was conducted exactly as described above except that the protease used was subtilisin from *B. licheniformis* (CAS 9014-01-1). Subtilisin concentrations between 0-1.785 U/mL were used and the equilibration buffer employed was defined fermentation medium without carbon source added, consisting of (g/l); Na-citrate dihydrate (2.28), $(NH_4)_2SO_4$ (10), KH_2PO_4 (7.4), K_2HPO_4 (13.6), $MgSO_4*7H_2O$ (0.8) and the following trace metals (mg/ml); $CaCl_2$ (2.2), Na_2MoO_4 (0.24), $FeSO_4*7H_2O$ (5.2), $ZnSO_4*7H_2O$ (7.2), $MnCl_2*4H_2O$ (10), $CuCl_2*2H_2O$ (0.85). For all adsorption isotherms, the data was fitted using the Langmuir model (Langmuir, 1918; Franzreb et al, 2007) and the Langmuir parameters for maximum binding capacity (Q_{max}) and dissociation constant (K_d) were estimated.

The non-specific binding of bovine serum albumin (BSA) to the magnetic adsorbents was also tested in a fermentation medium. For this test, standard medium for growing B. licheniformis, was chosen, which consisted of 5 g/L peptone, 3 g/L meat extract and 10 mg/L $MnSO_4*H_2O$, pH 7.0. In this case, 0.9 mg portion of bacitracin-linked magnetic adsorbents were washed three times with distilled and deionised water and added to 1 ml aliquot of BSA (0.5 mg/mL) in the medium. After 0.5 h of binding, the magnetic adsorbents were separated and the supernatants analyzed for the concentration of total protein.

The use of magnetic adsorbents in a fermentation broth requires their sterilisation. Preliminary experiments showed however, that the magnetic beads used in this study cannot be sterilised by autoclavation. The bead matrix appears to be damaged and changes colour from black to brown during this procedure. In addition the bacitracin peptide ligand is sensitive to the high temperatures employed (121 °C). Since magnetic beads have a similar size (~1-4 μm) to bacteria, filter sterilisation cannot be used. Thus sterilisation with ethanol was studied. The magnetic beads were soaked in 70% ethanol for 600 s and then washed three times in sterile distilled water before being spread on agar plates containing three different media, each chosen for enhancing the growth of bacteria, fungi and yeast respectively. The media were LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, pH 7.0), LB medium with added salts (0.52 g/L KCl,

0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH_2PO_4, 0.4 mg/L $CuSO_4 \cdot 5H_2O$, 0.04 mg/L $Na_2B_4O_7 \cdot 10H_2O$, 0.8 mg/L $FeSO_4 \cdot 7H_2O$, 0.8 mg/L $MnSO_4 \cdot 2H_2O$, 0.8 mg/L $Na_2MoO_4 \cdot 2H_2O$ and 8.0 mg/L $ZnSO_4 \cdot 7H_2O$) and YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose). After 3 days of incubation there was no visible growth on any of the agar plates. This method of magnetic adsorbent sterilisation was therefore used in all further work.

2.2.3 Cultivation conditions

An inoculum of *B. licheniformis* was prepared by growing the microorganism in 4 mL of standard medium in a test tube overnight. All of the innoculum was then added to 200 mL standard medium in a 500 mL baffled Erlenmeyer flask which was used in either of two ways: (1) immediately used to test the concept of HGMF for stabilising protease sensitive proteins during cultivation in the Erlenmeyer flask, or (2) incubated overnight and used as a pre-culture for inoculating fermenters. In the latter case, a sufficient volume of the pre-culture was added to a 1 L fermenter containing 800 ml of the standard medium to give an initial biomass concentration of 0.0015 g/L dry weight. All cultures were grown at 30°C and all Erlenmeyer flasks were shaken at 175 rpm on a reciprocal shaker.

For testing the concept of HGMF stabilisation in shake flasks, BSA was added after 20 hours of fermentation to give a concentration of 0.5 mg/mL. In fermentation (A) , a HGMF step followed immediately after BSA addition. In the control fermentation (B), no further steps were taken except for sampling. The concentration of BSA was chosen after conducting preliminary experiments, which showed (using SDS-PAGE) that a clear pattern of degradation could be seen over a 25 h period following incubation of BSA in sterile fermentation media containing Savinase added at a concentration to be expected during a fermentation of B.licheniformis (i.e. 0.2 NPU(S)/ml).

Cultivations in fermenters were conducted with stirring at 400 rpm, aeration at 800 ml/min, 30°C and pH 7. When the biomass concentration reached 0.16 g/L (i.e. after 18 h), BSA was added to the fermentation to give a concentration of 0.5 g/L. In fermentation (A), a HGMF step followed immediately after BSA addition, and again 12 h later. In the control fermentation (B), no further steps were taken except for sampling.

2.2.4 Magnetic adsorbent removal from Erlenmeyer flask culture

Immediately following BSA addition, 180 mg of magnetic adsorbents was added to the Erlenmeyer flask culture. The adsorbents were pre-disinfected by soaking for 10 min in a test tube containing 70% ethanol and washing three times with sterile MilliQ-water. After 30 min of incubation at 30°C and shaking at 175 rpm the adsorbents were aseptically removed from the fermentation broth using a magnetic filter. This was done by pumping the solution directly from the Erlenmeyer flask through an autoclaved 4.7 ml magnetic filter (linear flow velocity of 24 m/h) placed vertically in the 2.5 cm gap between the poles of a 0.32 T Steinert HGF-10 "on-off" permanent magnet separator (Steinert Elektromagnetbau GmbH, Köln, Germany) (Hoffman et al., 2002) and into another sterile Erlenmeyer flask using a peristaltic pump (Masterflex® Easy Load® model 7518-00, Cole Parmer Instrument Co., Vernon Hills, Ill, USA). The magnetic filter was 9 mm in diameter x 52 mm long and consisted of a glass tube packed with a stainless steel matrix (voidage ca. 0.9). The matrix (SS 430 type 9029, ~100 μm wire diameter) was obtained as a gift from KnitMesh (Surrey, U.K).

2.2.5 HGMF-fermenter system

The set-up for the HGMF process is sketched in Figure 2.1 and consisted of a 1 L fermenter connected to the magnetic filter and solution reservoirs via silicone tubing, 3-way valves, and a peristaltic pump (Masterflex® Easy Load® model 7518-00, Cole Parmer Instrument Co., Ill, USA). The complete system including buffers, reservoirs and magnetic filter (but excluding the magnetic adsorbents, pump and magnet) was assembled together with the fermenter and autoclaved. Each complete HGMF process consisted of a number of steps which will be briefly described. (1) The magnetic adsorbents to be used (sufficient for a concentration of 6.5 g/L in the fermenter) were suspended for at least 600 s in a 70% ethanol solution (for sterilisation) and then pumped from the particle reservoir through the magnetic filter with the field on, leading to adsorbent capture. Sterile water was then pumped into the system, replacing the ethanol and after switching off the magnetic field the adsorbents were released from the filter into the recycle loop to wash out entrained ethanol, before being pumped to the broth reservoir. Excess water was decanted aseptically from the adsorbents after magnetic settling. (2) At the appropriate time the contents of the fermenter were

pumped through the filter and into the broth reservoir, in order to flush the magnetic particles into the fermenter. Adsorption took place for 300 s in the fermenter before the broth containing the adsorbents was pumped through the magnetic filter with the field on and the broth collected in the broth reservoir (to avoid mixing with the broth in the fermenter), which took 360 s. The broth reservoir was held at 30°C with stirring. Immediately after the fermenter was emptied, it was refilled with the contents of the broth reservoir (i.e. now not containing magnetic adsorbents). (3) The magnetic adsorbents were then washed by flushing them out of the filter (field off) and into the recycle loop with washing buffer (100 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.5) and recycled for 600s. Subsequently the adsorbents were recaptured in the magnetic filter (field on) and the spent wash buffer sent to waste. The procedure was then repeated again with wash buffer and four times with cleaning buffer (5 mM succinic acid, 50 mM sodium tetraborate decahydrate, 1 mM $CaCl_2$, pH 6.0 mixed 1:1 with 1,2 propandiol) to ensure all bound proteins were removed. During cleaning, 20 min of recycle was used. (4) Finally the magnetic adsorbents were pumped back to the particle reservoir for sterilisation with 70% ethanol before being used again. The basic steps involved in HGMF processes have been described in detail previously and for more information the following should be consulted: Hubbuch et al. (2001), Meyer et al. (2005), Franzreb et al. (2007), Petersen (2007).

The box like (5.8 cm x 7.8 cm x 0.9 cm) stainless steel high-gradient magnetic filter (type Aa) used has been described earlier (Gomes, 2006; Ebner et al., 2007) and was positioned in the 0.32 T Steinert HGF-10 permanent magnet separator. The filter volume was 40 mL and it contained parallel magnetisable sheets of stainless steel 430 woven wire cloth (0.315 mm Ø wires, 1mm mesh), separated by spacers of stainless steel 316 woven wire cloth (0.9 mm Ø wires, 5 mm mesh) (Meyer et al., 2005; Gomes, 2006) both of which were obtained as gifts from Susan Venneker (Haver & Boeker, Oelde, Germany). The total volume of the filter and recycle loop was 195 ml and thus the adsorbent concentration during washing and cleaning was 22.1 g/L.

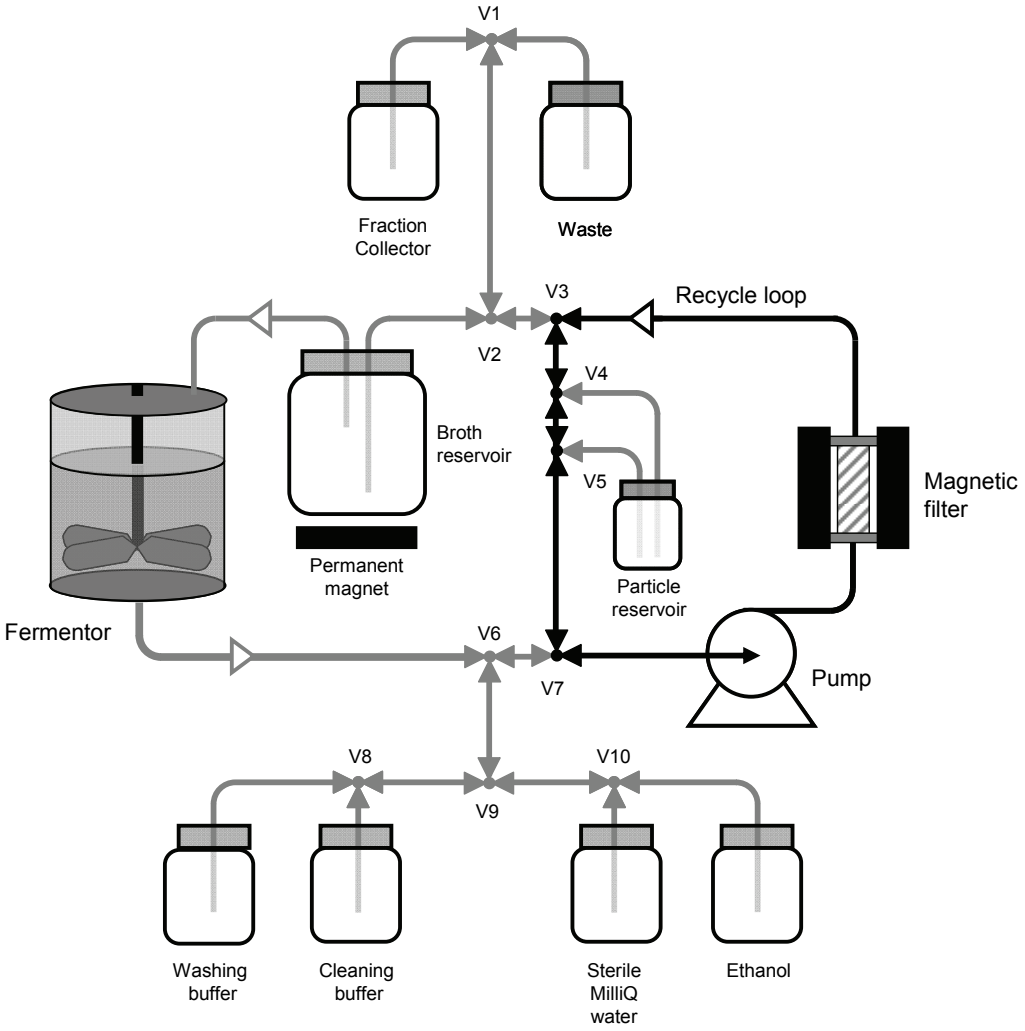

Figure 2.1: Schematic diagram of the coupled fermenter and HGMF process for in situ protease removal. A 1 L fermenter is connected via silicone tubing, 3-way valves (v1-v10), and a peristaltic pump to the magnetic filter and solution reservoirs. A recycle loop is formed by isolating the pump and filter with valves V3, V4, V5 and V7 to allow adsorbent sterilisation with 70% ethanol and washing and cleaning after use.

2.2.6 Analytical methods

Savinase was only used for characterising the adsorbents and the activity was measured using the assay described in Hubbuch et al. (2001). Briefly, the sample was added to buffer containing 11.18 g/L KCl, 19.7 g/L $Na_2B_4O_7 \cdot 10H_2O$, at pH 9.0 and 0.08 mg/ml N-Suc-Ala-Ala-Ala-p-nitroanilide substrate and the change in absorbance was measured at 405nm. All activities for Savinase were expressed as NPU(S)/ml, which is a Novozymes A/S internally defined unit. Purified Savinase with an activity of 4.21 KNPU(S)/g was obtained from Novozymes A/S and was used as the standard for all

analysis of Savinase activity. By measuring the concentration of total protein in the Savinase standard, using the BCA assay, it was found that 1 NPU(S) = 0.005 mg Savinase.

Total proteolytic activity as well as that of *B. licheniformis* subtilisin was evaluated by hydrolysis of the large substrate, azocasein as described by Fujimura and Nakamura (1987) with minor modifications. Briefly, 2 volumes of a 5% (w/v) azocasein solution in 0.05 M Tris/HCl buffer, pH 7.5 was mixed with 7 volumes of the Tris/HCl buffer and 1 volume of sample was added. Incubation was carried out at 37°C and after 0.5 h was immediately stopped by precipitation with 10 volumes of 10% (w/v) trichloroacetic acid. Samples were kept on ice for 0.3 h after which the precipitate was removed by centrifugation (360 s, 13,000 g, 4°C). Four volumes of the supernatant were then mixed with 1 volume of 1.8 M NaOH and the absorbance at 420 nm was measured. All activities were expressed in U/ml by relating to a standard curve constructed using subtilisin from B. licheniformis obtained from Sigma (CAS 9014-01-1). By measuring the concentration of total protein in the subtilisin standard, using the BCA assay, it was found that 1 U = 0.08 mg subtilisin.

The concentration of total protein was measured using a bicinchoninic acid (BCA) assay-kit from Pierce (Rockford, Ill, USA), which is based on the analysis method described by Smith et al. (1985). All protein concentrations were expressed as BSA equivalents.

Reducing SDS-PAGE was performed using 4-12% NuPAGE® Novex Bis-Tris gels from Invitrogen (Carlsbad, USA) by following the instructions from the manufacturer. Before sample treatment, the proteolytic activity in the samples was inhibited with phenylmethylesulfonyl fluoride (PMSF) by diluting 1:1 with a PMSF solution (45.5 g/L in isopropanol). The gels were stained with Coomassie Brilliant Blue (CBB R-250). Pictures of stained gels were taken on a CanonScan D660U (Canon Inc., Tokyo, Japan) or a Bio-Rad Gel-Doc 2000 fitted with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of the individual protein bands in the gels was carried out using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). On each gel a known concentration of BSA was loaded to permit normalisation of the other bands and for construction of a standard curve for protein concentration.

Prior to biomass determinations, any magnetic beads present in the samples were first removed by magnetic separation using a bar magnet. Then 6 ml of the fermentation broth was filtered through a pre-dried and pre-weighed 0.45 μm filter (PALL Corporate, NY, USA), washed 3 times with distilled water and dried in a microwave oven, before cooling in a dessicator and weighing.

The concentration of magnetic adsorbents in a sample were determined by first washing the sample with distilled water to remove salts, followed by drying of the beads in a pre-dried and pre-weighed glass tube at ~80° C to constant weight, and subsequently weighing.

2.3 Results and discussion

2.3.1 Binding properties of the magnetic adsorbents

The bacitracin-linked magnetic adsorbents are to be used for removal of trace amounts of troublesome proteases from a fermentation and it is thus important that they have suitable binding properties, i.e. low dissociation constants and high capacity. Furthermore, there exist certain constraints on the system. Binding must be acceptable at the pH values used in the cultivation and which are compatible with the magnetic adsorbents (the DVS coupling chemistry is unstable at pH > 8 (Hermanson et al., 1992)). Thus adsorption isotherms were first constructed with Savinase in buffer at pH 7.5 and the data was fitted to the Langmuir model (Langmuir, 1918). The results in Figure 2A show isotherms for a small 100 mg batch and for a large 4 g batch of adsorbents determined for both total protein and activity measurements. In all cases, good binding characteristics which are typical for a strong affinity interaction can be seen, namely a steep initial slope followed by a plateau region (Figure 2A). The values for the Langmuir parameters (i.e. Q_{max} and K_d) (Table 1) indicate that the adsorbents have properties which are similar to those reported previously by Hubbuch et al. (2001). The maximum binding capacities (Q_{max}) of the manufactured adsorbents are close to 200 mg/g, the dissociation constants (K_d) are in the micromolar range and the Q_{max}/K_d-values are approx. 5 L/g. It can thus be concluded that the magnetic beads fulfil the specifications of Franzreb et al. (2007) for efficient magnetic adsorbents suitable for bioprocesses (i.e. Q_{max} >> 100 mg/g, K_d in the sub-micromolar range and Q_{max}/K_d >> 5 L/g).

An adsorption isotherm for the binding of the subtilisin from B. licheniformis to magnetic supports was also constructed. In this case the adsorbents used were from a 36 g pool of nine individual 4 g batches, which had essentially identical binding characteristics. The subtilisin from B. licheniformis was chosen instead of Savinase for this study because fermentations were to be conducted with this organism and thus it was more relevant to choose this protease. Furthermore, a defined fermentation medium at pH 7 was chosen that would be compatible with both cell growth and the HGMF process. Preliminary experiments showed that the “crudeness” of the cultivation medium must be considered and that in particular large particles in suspension fouled

the magnetic filter which employed stacked 1 mm mesh sheets. The isotherm (Figure 2B) and the Langmuir parameters based on protein concentration for this isotherm (Table 1) show that the binding performance is similar to that seen for Savinase in buffer (i.e. Figure 2.2A). A Scatchard plot (Scatchard, 1949; Hubbuch and Thomas, 2002) of the adsorption data further confirmed that the adsorbents were highly specific for the subtilisin tested (Figure 2.2B). A straight line with a slope of -4.06 mL/U (i.e. K_d = 0.246 U/ml) and an intercept of 15069 mL/g (i.e. 15.07 L/g) was found (Table 2.1) suggesting that the bacitracin has a single specific binding site for the subtilisin and that non-specific binding of the subtilisin is minimal. These values are approximately the same as obtained from the Langmuir isotherm (Figure 2.2B) where $-1/K_d$ was -3.79 mL/U and Q_{max}/K_d was 14157 mL/g (value determined from protease activity isotherm). It can be concluded that the fermentation medium does not have a negative impact on the binding characteristics of the magnetic adsorbents.

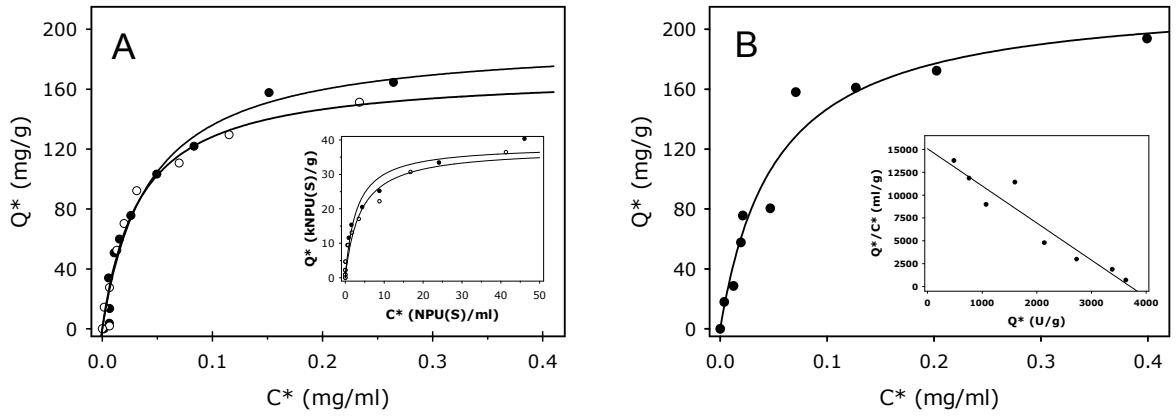

Figure 2.2: Adsorption isotherms fitted with the Langmuir model for the magnetic adsorbents constructed. (A) Savinase was employed in tris buffer with adsorbents from an initial 100 mg test batch (○) and from a scaled-up 4 g batch (●). The insert shows the same experiment but in which Savinase activity rather than total protein has been measured. **(B)** Subtilisin was employed in fermentation medium with a sample from a 36 g batch of pooled adsorbents. The insert shows the same experiment but for which activity measurements have been used to create a Scatchard plot.

The adsorption isotherms in Figure 2.2B can be used to determine the working capacity of the magnetic supports in a fermentation broth. If the goal is to remove all proteolytic activity from the fermentation, it can be seen from the isotherm in Figure 2.2B that the working capacity is ~60 – 80 mg/g (i.e. ~750 – 1000 U/g). The adsorption

kinetics of the magnetic supports was also investigated to define an appropriate binding time for protease removal.

Table 2.1: Langmuir parameters for the binding of protease to different batches of bacitracin-linked magnetic adsorbents.

	Q_{max}	K_d	Q_{max}	K_d [4]	Q_{max} / K_d [4]
	10^4 NPU(s)/g	NPU(S)/mL	mg/g	10^{-6} M	L/g
Present work, 100 mg batch[1]	3.7	3.8	170.8	1.24	5.14
Present work, 4 g batch[1]	3.8	2.8	193.3	1.58	4.59
Present work, 36 g batch[2]	0.37 (0.37)[5]	0.26 (0.246)[5]	222.8	1.94	4.3 (15)[5]
From Hubbuch et al., 2001[3]	1.8	2.5	92.8	0.88	3.95

[1]Binding conducted in 100 mM Tris, 20 mM $CaCl_2$ buffer pH 7.5 with added Savinase. [2]Binding conducted in fermentation medium with added subtilisin, activity expressed in U not NPU. [3]Binding conducted with sterile diluted cell free *B. clausii* fermentation broth containing produced Savinase. [4]Based on protein concentration measurements and calculated using a molecular weight of Savinase of 26698 Da (Personal communication with Dr. Jesper Brask, Novozymes A/S, Bagsværd, Denmark). [5]Parameters from Scatchard plot. Note: values based on Subtilisin activity not protein concentration

Binding was extremely rapid and it was found that 88% of the equilibrium concentration of subtilisin bound to the adsorbents was adsorbed after only 30 s. Furthermore, equilibrium was reached at 85% of the starting concentration after only 300 s (results not shown). Similar rapid binding kinetics has been reported for other magnetic adsorbents and is believed to arise from their non-porous nature with respect to proteins. For example, Gomes (2006) found that the time needed for binding 0.15 g/L added trypsin to 5.5 g/L benzamidine-linked adsorbents in crude cheese whey was 240 s.

The Langmuir parameters obtained for the manufactured adsorbents show that Savinase and other subtilisins produced in a fermentation broth will be adsorbed specifically to the magnetic beads when they are added to the broth. Proteins are however “sticky” of nature and given that BSA was to be used as the model product, it was relevant to determine whether there was non-specific binding of BSA to the magnetic adsorbents. It was found that when using the same BSA concentration as to be added to the fermentation broth (0.5 mg/mL) only 7% of the added BSA was bound when magnetic beads (0.9 mg/mL) were added and removed again (results not shown), which can be expected to have little impact on the later results.

2.4 Use of HGMF to avoid proteolytic degradation of BSA during fermentation

2.4.1 Cultivations in shake flasks

Shake flask cultivations were used first to test the concept of removing proteases during *Bacillus licheniformis* fermentations. The protein BSA was added 20 hours after inoculation followed immediately by removal of proteases from the fermentation broth using HGMF. This treatment led to stabilisation of the BSA for 7 h following its addition (Figure 2.3A) and the protein was not totally degraded until 13 h had elapsed. In contrast, BSA vanished within 7 h in the control fermentation not subjected to magnetic adsorbents (Figure 2.3B). Examination of the fermentation data showed that the protease activity was instantaneously reduced by 60% following the HGMF treatment.

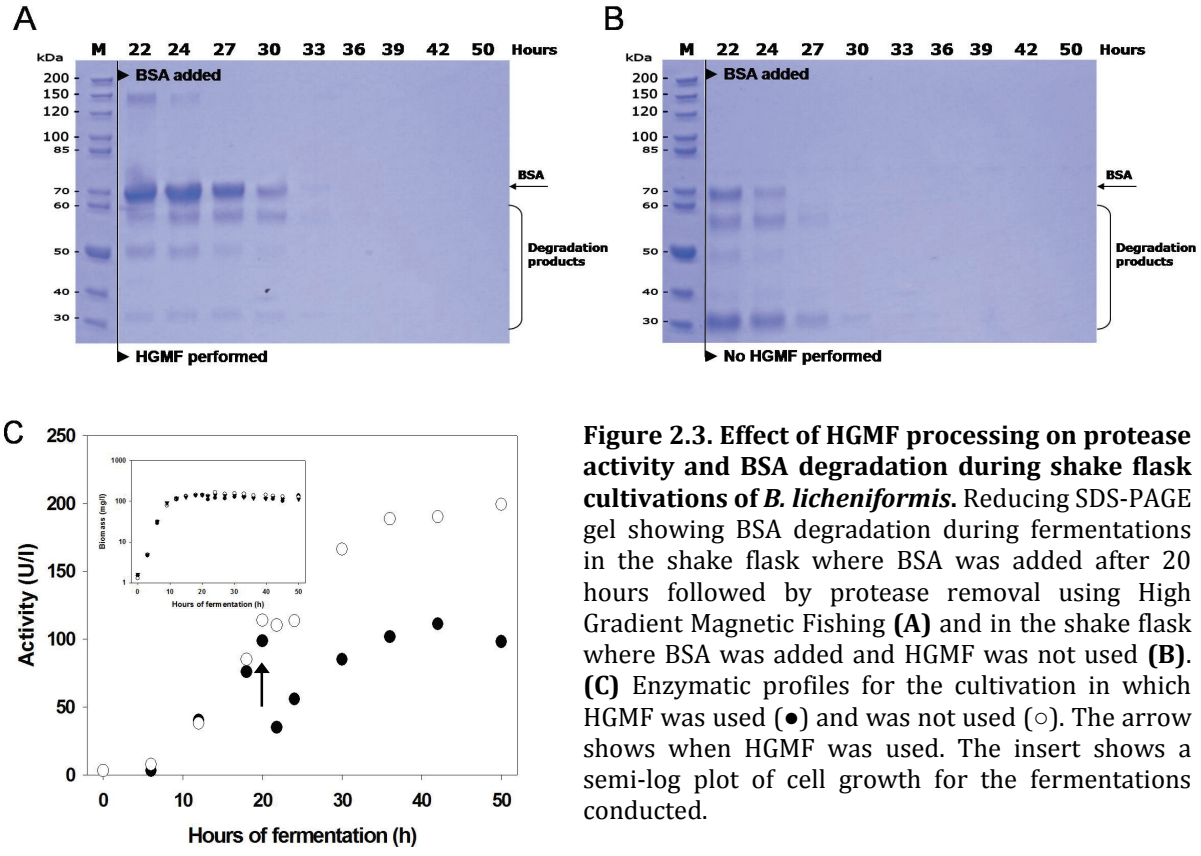

Figure 2.3. Effect of HGMF processing on protease activity and BSA degradation during shake flask cultivations of *B. licheniformis*. Reducing SDS-PAGE gel showing BSA degradation during fermentations in the shake flask where BSA was added after 20 hours followed by protease removal using High Gradient Magnetic Fishing **(A)** and in the shake flask where BSA was added and HGMF was not used **(B)**. **(C)** Enzymatic profiles for the cultivation in which HGMF was used (●) and was not used (○). The arrow shows when HGMF was used. The insert shows a semi-log plot of cell growth for the fermentations conducted.

However, given that there was little cell removal by HGMF (Figure 2.3C insert), the protease continued to be produced at a similar rate to the control fermentation, explaining why the added BSA was degraded after the HGMF treatment. A number of subsequent HGMF steps thus appear necessary to keep the protease activity at a constant low level in order to totally avoid the degradation of BSA.

2.4.2 Cultivation in fermenters

Fermentations of *B. licheniformis* with added BSA and two cycles of HGMF processing were carried out in fermenters and compared to those in which HGMF was not used. In both cases the BSA was added 18h after inoculation, when the biomass concentration reached 0.16 g/L and protease activity was similar ~0.05 U/ml. The results showed that two cycles of HGMF spaced 12 h apart kept the protease activity low (~ 75 U/L) throughout the whole cultivation whereas enzyme activity continued to increase (up to ~ 200 U/L) in the control experiment (Figure 2).

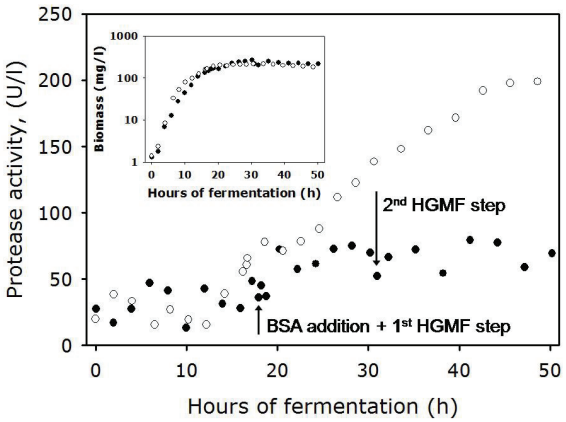

Figure 2.4: Comparison of the effect of HGMF processing on protease activity during a cultivation of *B. licheniformis* (●) with a cultivation in which HGMF was not used (○). One liter working volume fermenters were used and in both cases BSA was added after 18 h and before an eventual HGMF process. The arrows show when HGMF was used. The insert shows a semi-log plot of cell growth for the fermentations conducted.

The degradation of BSA due to the proteases in the fermentation broths was followed for both fermentations and visualised using SDS-PAGE (Figure 2.5). From the SDS-PAGE gels the effect of the two HGMF steps is clear. The degradation of the BSA was greatly reduced when HGMF was used (Figure 2.5A) and after 32 hours a very strong band corresponding to 55% of the original BSA could still be observed (Figure 5A and C) due

to the lowered proteolytic activity. In contrast, when no HGMF was carried out the BSA was fully degraded over 17 hours (Figure 2.5B and 2.5C). From the results obtained it can be concluded that using HGMF reduces the protease activity and thereby stabilises BSA. Furthermore, that in order to keep the protease activity low throughout the cultivation, repeated use of HGMF is necessary since proteases are constantly being produced.

The use of HGMF had little effect on cell concentration, suggesting few cells were removed by the procedure and the biomass profiles throughout the fermentation were similar (figure 2.4, insert). The maximum specific growth rates were 0.51 h^{-1} and 0.44 h^{-1} for the cultivation with HGMF and without HGMF, respectively. Observations of the cells using a microscope in samples taken before, during and after the 1st HGMF step showed no visible differences in morphology and that use of magnetic adsorbents directly in the fermentation broth had no obvious deleterious effect. Furthermore microscope pictures of samples taken during the 1st HGMF step, while the magnetic adsorbents were inside the bioreactor, showed that the cells did not adhere to the magnetic beads or cause their aggregation.

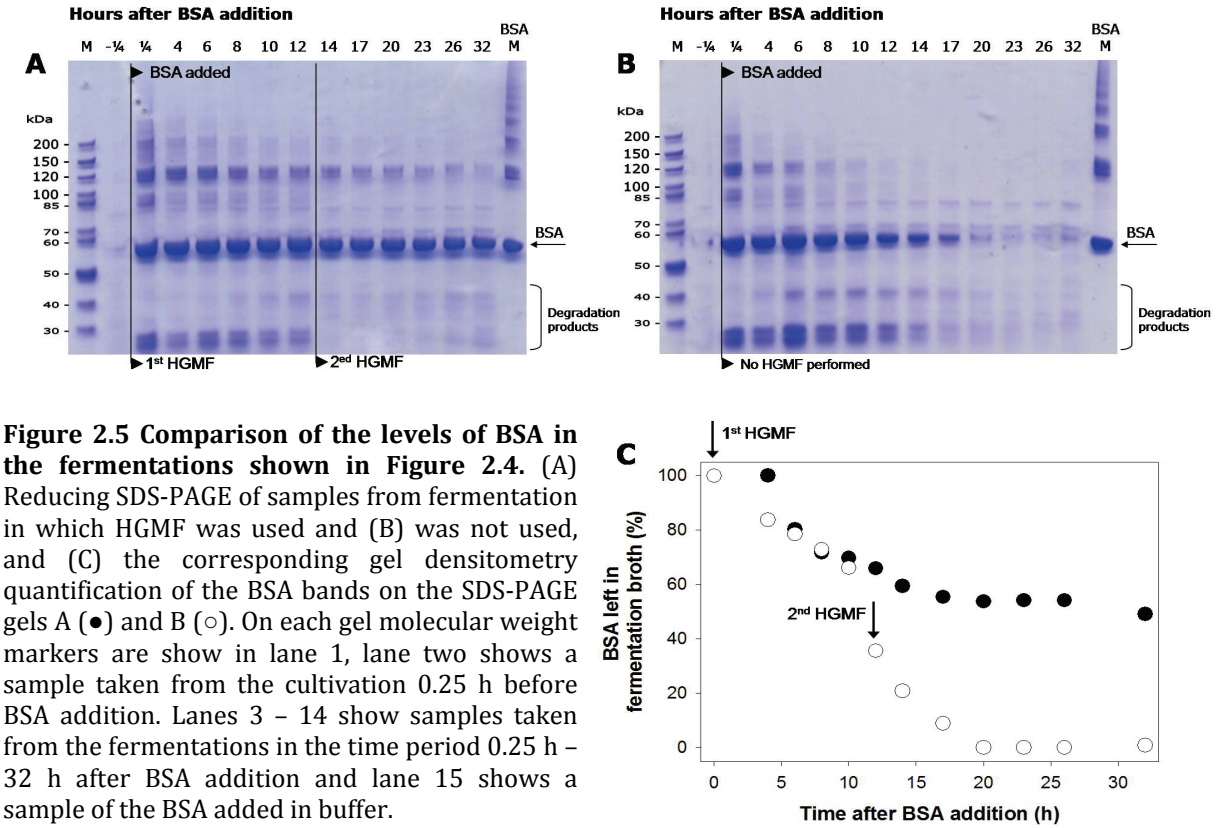

Figure 2.5 Comparison of the levels of BSA in the fermentations shown in Figure 2.4. (A) Reducing SDS-PAGE of samples from fermentation in which HGMF was used and (B) was not used, and (C) the corresponding gel densitometry quantification of the BSA bands on the SDS-PAGE gels A (●) and B (○). On each gel molecular weight markers are show in lane 1, lane two shows a sample taken from the cultivation 0.25 h before BSA addition. Lanes 3 – 14 show samples taken from the fermentations in the time period 0.25 h – 32 h after BSA addition and lane 15 shows a sample of the BSA added in buffer.

It could be argued that a less labour and process intensive alternative to having a series of sequential HGMF steps during a cultivation could be to add magnetic adsorbents to the bioreactor from the beginning, leaving them in the broth, and then to carry out one HGMF step at the end. It is however, expected that leaving the adsorbents inside the bioreactor will not significantly diminish proteolysis of the product. In work conducted by Gomes (2006) with benzamidine-linked affinity magnetic adsorbents it was shown that allowing the adsorbents to stay in a solution of whey containing trypsin did not halt proteoloysis. On the other hand, when the experiment was repeated and the magnetic adsorbents were added to adsorb the trypsin and then removed from the solution by HGMF, the hydrolysis was immediately stopped. The reasons for the inability of magnetic adsorbent immobilised inhibitors to stop hydrolysis reactions should be the subject of further work. The spectrophotometric assay methods developed by Schultz et al. (2007) for investigating magnetic particle immobilised enzyme activity could possibly be adapted to study the kinetics of free enzymes in the presence of immobilised inhibitors.

2.5 Conclusions

In conclusion, it has been found that HGMF can be used to remove trace amounts of troublesome proteases from fermentation broths during cultivation. The protease activity could be kept at a minimum throughout the cultivation of *B. licheniformis* thereby reducing proteolytic degradation of other proteins present in the broth. Contrary to commonly used techniques for avoiding degradation of interesting extracellular peptide and protein products during cultivation, HGMF has been found to have the potential to be an easy and efficient in situ technique. It is expected that compared to other alternatives, the advantages of using HGMF during a fermentation to avoid proteolysis are: That it can be conducted *in situ*, the cultivation is not disturbed, the product not damaged and that the magnetic adsorbents can be reused for many repeated cycles of protease removal.

Acknowledgements

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Chapter 3

Setup and characterization of a HGMF-based process for semi-continuous PEGylation

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3. Setup and characterization of a HGMF-based process for semi-continuous PEGylation

3.1 Introduction

As the application of poly peptide based biocatalysts, enzymes and pharmaceuticals has e xpanded, so has the search for methods to enhance their activity and stability, and one of the most successful techniques is known as PEGylation. The term PEGylation describes the procedure by which polyethylene glycol (PEG) is covalently attached to poly peptides, and this may lead to altered physiochemical properties including improved solubility in aqueous solution and in some organic solvents (Abuchowski et al., 1977; Ljunger et al., 1993; Roberts et al., 2002). In addition to this, PEGylated drugs have also been shown to display decreased immunogenicity as well as greatly enhanced pharmakinetic profiles as a result of reduced renal excretion, proteolysis and opsonization (Hamidi et al., 2006). Since the first PEGylated drug, ADAGEN (Enzon Pharmaceuticals) was approved by the FDA in 1990 to treat severe combined immunodeficiency, several others have been introduced into the market. For example two antiviral interferon-based drugs sold under the commercial names PEGasys (F.Hoffmann-La Roche) and Peg Intron (Schering-Plough) as well as the drug Certolizumab pegol (Cimzia UCB) used to treat Crohns disease. The latter contains a PEGylated Fab fragment of a humanized TNF inhibitor monoclonal antibody (Chakravarti et al., 1991; Keating and Curran, 2003; Melmed et al., 2008; Pedder SC, 2003).

The most commonly used method employed when performing PEGylation, involves a simple procedure by which the target molecule in free solution, is mixed with a reactive PEG agent. Although advances in PEGylation chemistry have improved the specificity of the reaction, the approach still lacks tight control. The lack of control may result in undesirable conjugate profiles with too many or too few attached PEG groups, loss of activity caused by modification of the active site or amino acids in the vicinity and even cross-linking of the target. These problems reduce yield and complicate purification of the desired PEG-conjugate. To overcome the problems associated with PEGylation of freely suspended targets, temporary immobilization during the reaction might be one

solution. Just such an approach was studied by Caliceti et al (1993). Those authors bound trypsin via the active site to the inhibitor benzamidine, which had been immobilized on a large, porous insoluble resin similar to that used in chromatography. Subsequently the PEGylated trypsin could be released from the resin and recovered. The authors found that the method produced a PEG-conjugate with higher activity than trypsin which had been PEGylated whilst in free solution, which was speculated to be due to protection of the active site by immobilization. Recently, this approach was taken a step further by Petersen (2007) who demonstrated that the PEGylation of immobilized trypsin could also be performed successfully by using a micron-sized benzamidine derivatized magnetic support in batch reactions (Figure 3.1). Petersen (2007) showed that the unique properties of the magnetic support allowed the time of exposure to the activated PEG to be tightly controlled. The reactions were done in 1-2 ml scale and the immobilized PEGylated trypsin could be rapidly removed from the unreacted PEG using a bar magnet. Subsequently the trypsin-PEG-conjugate could be released and recovered. Petersen (2007) suggested that extension of her work to a HGMF system, analogous to that used earlier by Ferre (2005A/B), may permit scalable continuous protein PEGylation under tight control.

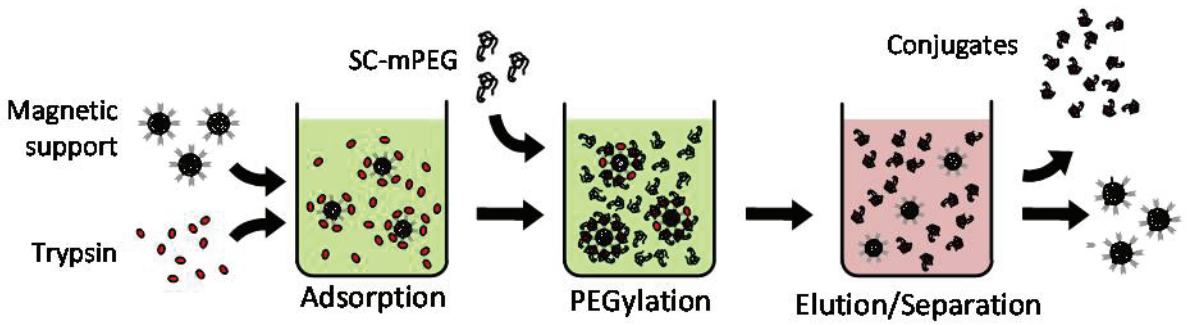

Figure 3.1: The batchwise PEGylation process used by Petersen (2007). The process of PEGylation using magnetic supports was conducted by performing a three step serial process of adsorption, PEGylation and wash/elution. During adsorption, the target protein (trypsin) was adsorbed on the surface of a non-porous magnetic support, while the actual covalent attachment of PEG groups was achieved by reaction with succimidyl carbonate monomethoxy polyethylene glycol (Sc-mPEG). Finally during the separation/elution step excess PEG was removed, the PEG-conjugates eluted and separated from the magnetic support.

Cationic bovine trypsin (EC. 3.4.21.4) belongs to the class of serine proteases and has a molecular weight of 23.8 kDa in the active form. It is expressed and secreted in pancreas as an inactive precursor known as trypsinogen, and during maturation a terminal hexapeptide is removed and the chain is autoproteolytically cleaved into two chains with molecular weights of approximately 11 and 13 kDa, respectively. The two chains are connected through a number of disulfide bonds (Walsh, 1970) and the primary sequence of the matured trypsin, denoted β-trypsin, contains 14 lysine residues which together with the two N-terminals makes up 16 potential PEGylation targets (shown in figure 3.2B). The ligand benzamidine (shown in figure 3.2A) is a reversible competitive inhibitor, and can thus bind trypsin via the active site by resembling the natural substrate arginine. The dissociation constant for both ligand and protein in free solution is around 18 μM at pH 8.15 (Barmann, 1969) and the ligand is shown in figure 3.2B. Attachment of the ligand to a solid support and selective binding of trypsin is a classic affinity adsorption system that is well characterised. The manufacturing of the base-matrix and the subsequent coating with polyvinyl alcohol and derivatization with benzamidine were also well established.

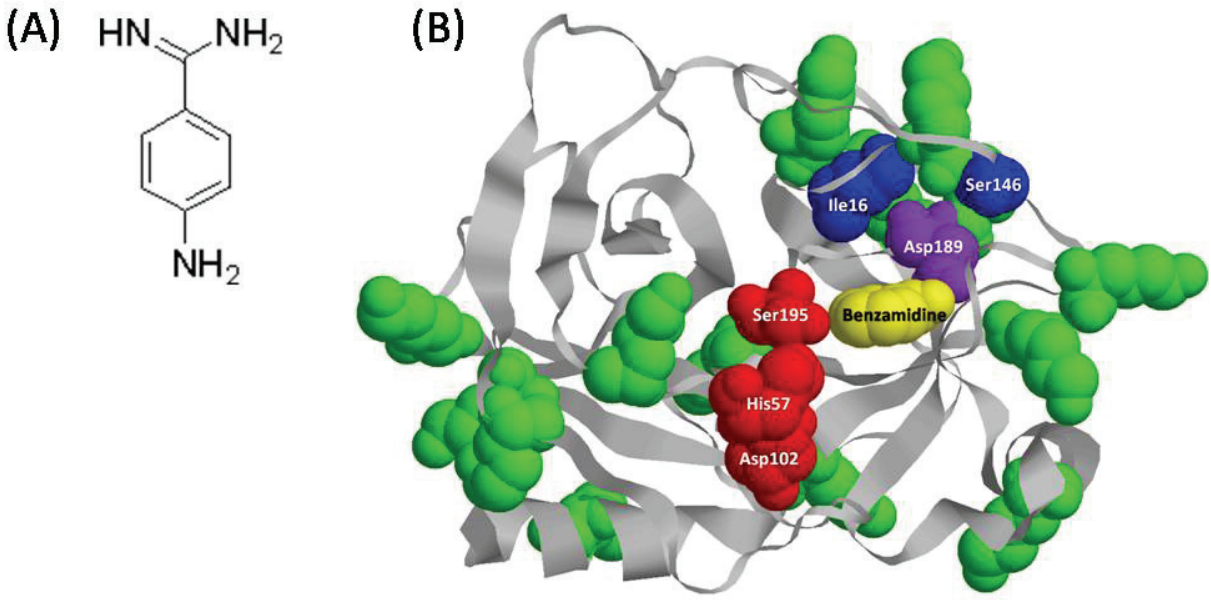

Figure 3.2: The benzamidine/trypsin affinity system. (A) Molecular structure of 4-aminobenzamidine, (B) Three dimensional structure of the benzamidine bovine pancreatic ß-trypsin complex (PDB no: 1CE5). Benzamidine (ligand) is coloured yellow and the residues important for the enzymatic activity (His57, Asp102, Ser195) or for binding (Asp189) is labelled and shown in red or purple respectly. The N-terminal residues (Ile16, Ser146) is coloured blue, while the 14 lysine residues (58, 84, 104, 106, 140, 151, 154, 164, 185, 201, 215, 217, 223, 232) making up the primary PEGylation targets are coloured green. The structure is visualized using the RasWin version 2.7.4.2 (Dowling College, ARCiB Project, Kramer Science Center KSC020, Oakdale, NY 11769 USA).

The aim of the work presented in this chapter was to develop a HGMF-based semi-continuous PEGylation process. The process should have performance comparable to that possible with small-scale batchwise microtube based reactions employing magnetic supports, with respect to the type of PEG-conjugates formed, concentrations of reactants required, reaction times and in particular the ability to control the PEGylation profile (the number of PEG groups attached). To achieve these goals, each step in the process, including adsorption of the target protein to the magnetic support, and the PEGylation process itself (including termination of the reaction) were investigated first. Then the results were used to design the process, which was subsequently characterized.

3.2 Materials and Methods

3.2.1 Chemicals and materials

Iron(II) chloride tetrahydrate (44939), Iron(III) chloride hexahydrate (12319), 3-aminopropyltriethoxysilane, 50% grade I glutaraldehyde (G7651), allyl glycidyl ether, 4-aminobenzamidine hydrochloride hydrate (857661), sodium tetraborate (229946), lysine hydrochloride (L5626), bovine pancreas trypsin (T8003), Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (B4875), N-bromo-succinimide (NBS) were all obtained from Sigma-Aldrich (St. Louis, Mo, USA). 2 kDa succimidyl carbonate monomethoxy polyethylene glycol (Sc-mPEG) was kindly supplied by Novozymes A/S (Bagsvard, Denmark). Neodymium block magnets were obtained from Danfysik A/S (Jyllinge, Denmark) and Hindsbo magneter Aps (Roskilde, Denmark), and magnetic racks were made in-house.

3.2.2 Manufacturing of micron-sized non-porous magnetic particles

Benzamidine coupled non-porous magnetic supports were routinely prepared using previously reported methods and are described briefly below. The aminosilane terminated magnetic base matrix, prepared from iron(II)/iron(III) chlorides and 3-aminopropyltrietoxysilane, were manufactured according to the procedures described by Hubbuch et al. (2002). Polyglutaraldehyde coating was then conducted as described by O'Brien et al. (1996), while subsequent attachment of the allyl glycidyl ether link, bromination using NBS and coupling of 4-aminobenzamidine were carried out as explained by Heebøll-Nielsen (2002). Scale up of the manufacturing procedure from 100 mg to 1 g was carried out according to Petersen (2007). To ensure the quality of each batch, the binding characteristics were determined using batch binding studies as described previously (Petersen, 2007). Briefly, known masses of adsorbents (ca. 2 mg) were mixed with different known concentrations of the protein to be bound. The mixtures were then incubated with shaking at room temperature, the adsorbents then magnetically settled and the concentration of protein (or enzyme activity) in the supernatant determined. The data was plotted and the Langmuir model fitted using Sigma Plot 6.0 (SYSTAT Software Inc, Chicago, IL, USA) to determine the maximum binding capacity (Q_{max}) and dissociation constant (k_d). Size-distributions for the

supports produced were also determined using a Mastersizer 2000 (Malvern Instruments Ltd Worcestershire, UK). Particles were stored in storage buffer (20 mM sodium phosphate, 1 M NaCl, pH 6.8) at 4°C until required.

3.2.3 Manufacturing of the Staggered Herringbone Mixer (SHM) type reactors

The reactors (Figure 3.3A-C) were fabricated as long channels, with mixing structures to facilitate constant mixing of magnetic particles and liquid in much the same way as is performed in an eppendorf tube on a vortex mixer. The staggered herringbone mixer (SHM) principle was proposed and demonstrated by Stroock et al. (2002) and allows efficient mixing without any active elements. The liquid flow in itself provides the mixing as is seen in Figure 3.3D. In the demonstration, constant flows of fluorescent and non fluorescent liquids are pushed through two inlets and into the SHM reactor. At the start of the reactor it can be seen that the fluorescent liquid is in one side of the channel and the non fluorescent liquid in the other side of the channel. Further down the channel the two liquids are completely mixed.

The reactors were fabricated in 5mm thick transparent and black PMMA purchased from Nordisk Plast (Assentoft, Denmark). The transparent PMMA substrate was structured using a CO_2 laser ablation system commercially available from Synrad (Mukilteo, WA, USA). A study and explanation of how the system works can be found elsewhere (Snakenborg D et al. 2004). The structured substrate (Figure 3.3a) was then bonded to an unstructured black PMMA substrate. The laser used for the bonding was an FLS IRON laser aperture (IRON 30/810-LH044 diodelaser, FT-163 F-Theta objective, IRON PSIF control unit, Fisba Optik AG, St. Gallen, Schwitzerland). The laser bonding setup and a partially bonded SHM reactor are shown in Figure 3.3b and c. Minstac 062 fittings were used for connection to the SHM mixers and were purchased from The Lee Corporation (Westbrook, CT, USA). Further reading on the manufacturing and performance of the SHM-type reactors may be found in Lund-Olesen (2008). The SHM units prepared and used in the current study are listed in Table 3.1.

Table 3.1: SHM-type reactors prepared and used in this study

Designation	Applied function	Measured internal volume (ml)
SHM-1A	Flow testing (transparent)	1.0
SHM-2A	Adsorption	1.0
SHM-2B	PEGylation	2.0
SHM-3A	Termination/Adsorption	0.5

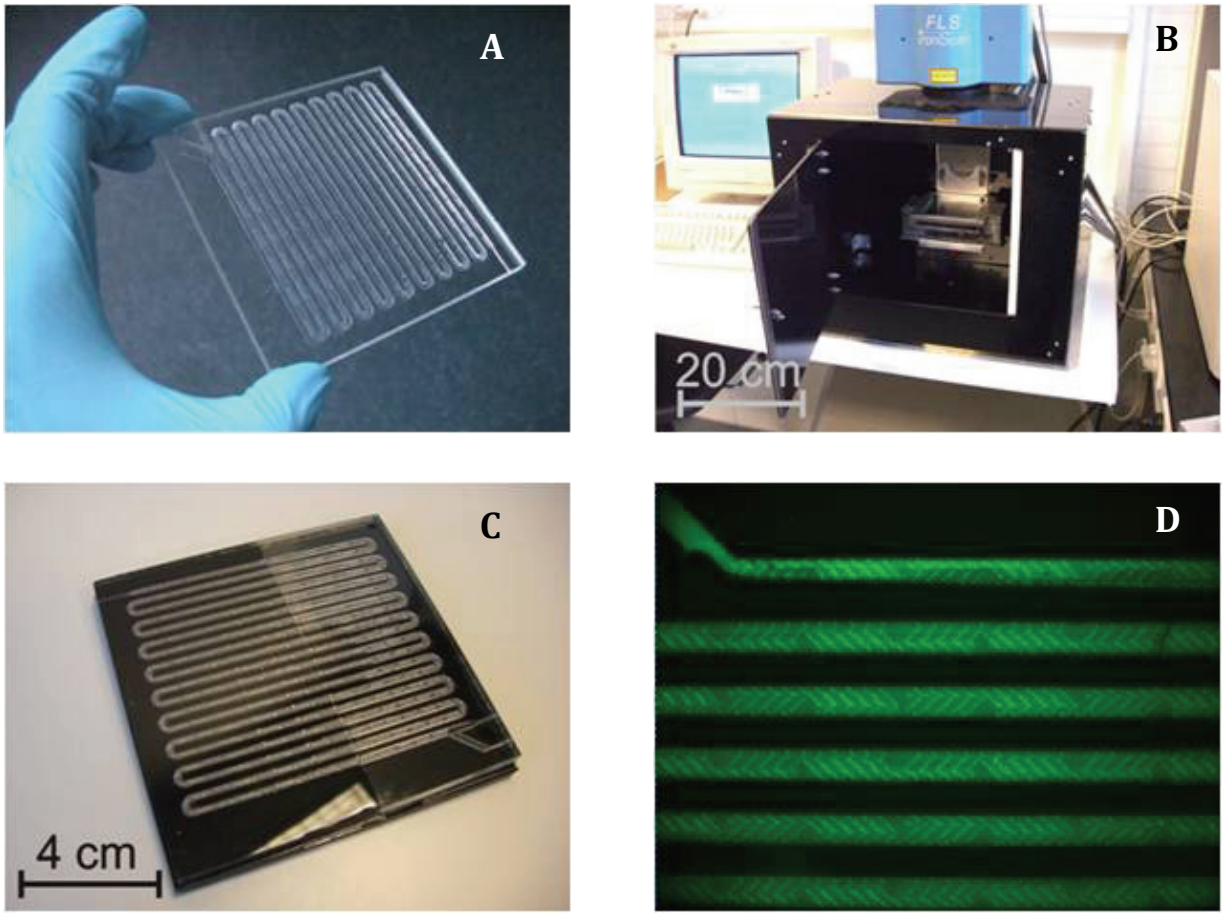

Figure 3.3: Preparation of the Staggered Herringbone Mixer units. (A) PMMA substrate with structures for the SHM reactors. (B) Laser bonding setup. (C) Half bonded SHM reactor (D) Fluorescence micrograph from an experiment where fluorescein isothiocyanate (top channel) and non fluorescent milliQ water (Bottom channel) are pumped into an SHM reactor at constant flow rates leading to rapid mixing of the two streams and the observation of fluorescence over the whole channel width.

3.2.4 Small scale adsorption and PEGylation experiments

For small scale trials, the following general procedure was used. The trypsin was first adsorbed to the magnetic support in a batch binding reaction and after removal of unbound trypsin, activated PEG was added and the reaction allowed to proceed as a batch reaction.

Adsorption: Investigation of the adsorption of trypsin onto the benzamidine derivatized magnetic support was performed using a common approach. Fresh magnetic support (i.e. not previously used) was prepared by removing the storage buffer, washing the particles three times with PEG4 buffer (100 mM $Na_2B_4O_7$ adjusted to pH 8 and supplemented with 10 mM $CaCl_2$), or the relevant buffer as mentioned in the text, and resuspended to a concentration of 8 mg/ml. For each batch binding experiment 250 µl of the particle suspension was mixed with 250 µl of the trypsin solution in question, normally between 0 and 4 mg/ml. To ensure efficient mixing, the microtubes used in the adsorption study were placed on a shaker operated at ca 300RPM (IKA Vibramax-VXR, IKA-Werke GmbH & CO. KG, D-79219 Staufen, Germany) and the incubation period was 30 minutes unless otherwise noted (30 minutes has previously been shown to be more than sufficient time to reach equilibrium by Gomes (2006), Heebøll-Nielsen (2002), Hubbuch (2000) and Pedersen (2007)). In order to collect a sample for analysis, the tube was placed in a magnetic rack for 2 minutes after which a sample was rapidly removed using a pipette. Before analyzing the sample it was spun at 13000 rpm in a micro centrifuge for 1 minute to make sure that there were absolutely no traces of the support, which could potentially interfere with the BCA protein assay. For studies of adsorption kinetics, the method described above was used to study adsorption periods of 15 and 30 minutes. However for the study of shorter periods more rapid separation was needed. Thus in the case of the 2.5 or 5 minute binding studies a centrifuge was used to achieve rapid separation of the loaded magnetic adsorbents by spinning the sample at 13000 rpm for 30 seconds in a centrifuge, after which support free samples were rapidly removed with a pipette. The 1 minute adsorption experiments were performed by mixing supports and the trypsin solution using a Vortex mixer for exactly 45 seconds followed by centrifugation and sample isolation as previously described.

PEGylation: Studies of PEGylation were performed using magnetic supports preloaded with a high trypsin concentration (2 mg/ml), which was known to give a loading of approximately 190 µg trypsin/mg support (see Figure 3.6). Subsequently 500 µl of 2 mg/ml loaded magnetic support was added to a 2 ml microtube and 500 µl of activated PEG dissolved in PEG4 buffer (2x concentration) was added to the particles and timing of the reaction initiated. During early studies in the current work, the PEGylation reaction was stopped by rapidly diluting the 1 ml reaction solution by addition to a 15 ml falcon tube containing 10 ml PEG4 buffer. In later studies a method involving addition of 1 volume 50 mM lysine to 1 volume of PEGylation reaction was used to block the covalent attachment of PEG, thus stopping the reaction. A bar magnet was subsequently placed on the side of the tube containing the reaction and the magnetic support carrying the immobilized conjugates separated from the supernatant. The support was then washed at least three times for 5 minutes with PEG4 buffer under vigorous shaking to remove excess PEG. The conjugates were finally eluted from the magnetic support by resuspending in 250 µl elution buffer (100 mM glycine pH 2.6, 10 mM $CaCl_2$) and incubated with vigorous shaking for at least 10 minutes. The final step of separating the particles from the supernatant was performed using a 0.15 Tesla magnetic rack (manufactured in-house).

3.2.5 System setup for the HGMF-based semi-continuous PEGylation

The semi-continuous PEGylation process was based on three serially connected steps denoted adsorption, PEGylation, and elution/separation and a schematic of the process is shown in Figure 3.1. The magnetic particle suspension and the trypsin solution were both supplied to the system using a dual syringe pump (Harvard Syringe pump Type 22, Harvard Apparatus, Holliston, Massachusetts 01746, USA) able to handle syringes of different sizes (1 to 25 ml) and to precisely control the flow. The syringe pump was placed on a shaking table (ca. 50 rpm) which in combination with glass beads[2] present in the syringe served to ensure that the magnetic particles remained well mixed. The syringe pump initiates the process and the particle suspension and the trypsin solution were led via 1 mm Teflon-tubing to the first SHM-type reactor (SHM 2A-1 ml), where the

[2] The size and number of the added glass beads varied according to the size of the syringe. In all cases the chosen diameter served to ensure that the glass beads were retained in the syringe and that enough turbulence were created to retain a uniform suspension based on visual inspection.

flows were combined and mixed as they travelled through the channel system permitting adsorption of trypsin (step 1). As the flow left the absorption step it continued through a short stretch of Teflon tubing to the next SHM-type reactor (SHM 2B-2 ml) in which a flow of activated mPEG was added to the mixture by a reciprocal pump (Pharmacia P-500, GE Healthcare, Chalfont St. Giles, UK). The liquid was led through the channel system in the SHM reactor allowing the PEGylation to take place. The solution then passed immediately to the last of the SHM-type reactors (SHM 3A-0.5 ml) where a lysine solution was supplied by another reciprocal pump causing (Pharmacia P-500, GE Healthcare, Chalfont St. Giles, UK) the PEGylation reaction to terminate by means of competitive reaction with the free amino groups on the lysine.

The flow then proceeded into a loop designed to enable semi-continuous HGMF based washing, separation and elution of PEG-conjugates. The loop was prepared using approximately 60 cm silicon tubing with a 6 mm diameter and was connected to a 5/16" inline static mixer (KH-04668-12, Cole-Parmer, Ill, USA) and a magnetic filter unit. The magnetic filter was prepared from a cone shaped polyethylene tube with an approximate volume of 7 ml packed with approximately 2.5 g stainless steel matrix (SS 430 type 9029, received as a gift from KnitMesh Ltd., South Croydon, Surrey, UK). A peristaltic pump (Verderlab CD-10, Verder Ltd, Leeds, UK), was used in the HGMF process, and within the loop (Figure 3.4A) four 3-way valves were also placed, which served to enable regulation of the flow in and out of the system. To investigate the performance of the different parts of the total HGMF-based semi-continuous system, two additional systems were used. The first system (Figure 3.4B) was used to evaluate adsorption using either SHM3A or SHM2A mixers and the flow was collected using a simple bar magnet placed on the side of a 14 ml tube. The second setup (Figure 3.4C) was used to investigate the PEGylation process and was built around a SHM 2B mixer. In this case the samples were collected directly into a 14 ml tube containing 50 mM lysine and washing, separation and elution were performed after all samples were collected. Prior to analysis the samples were stored on ice and subsequent analysis was carried out within 2-3 hours.

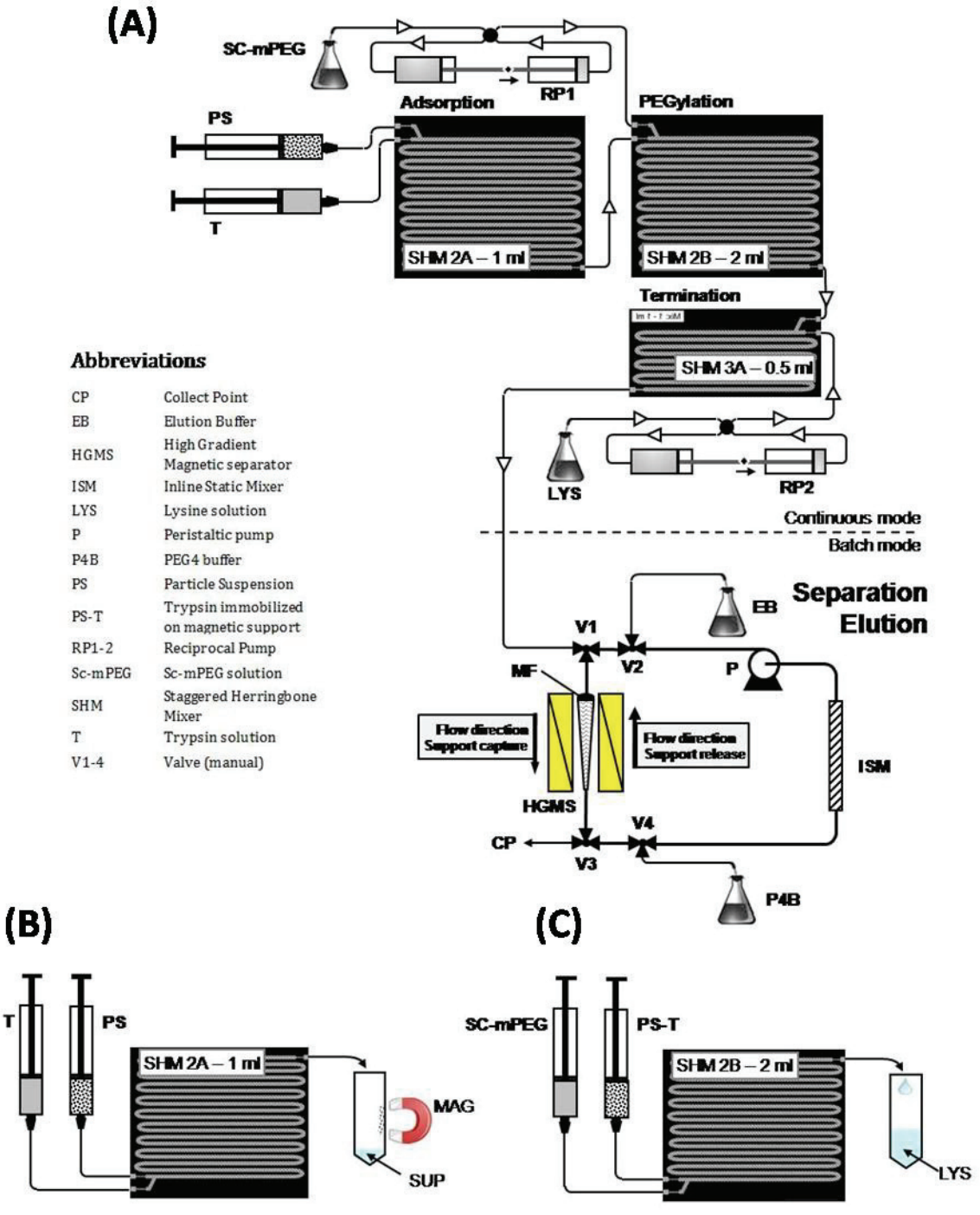

Figure 3.4: Schematic overview of the HGMF-based semi-continuous PEGylation process: (A) HGMF-based semi-continuous PEGylation process. (B) Setup designed to analyze adsorption using either the SHM-type 2A (1 ml) or 3A (½ ml). (C) Setup used to analyze performance of the SHM-type 2B unit during the PEGylation step.

3.2.6 Operation of the HGMF-based semi-continuous PEGylation system

Adsorption: During full scale operation of the HGMF-based PEGylation system the shaking table was set at 150 rpm to ensure that the magnetic support contained in the syringe was kept in suspension. The pumping velocity of the syringe pump was 125 µl/min resulting in a total combined flow rate of 250 µl/min during the adsorption step, which was performed with a particle concentration of 2 mg/ml and a trypsin concentration of 0.6 mg/ml. The process time during the adsorption step was 5.12 minutes.

PEGylation: The PEGylation step was achieved using various concentration of SC-mPEG. The activated PEG solution was injected into the process flow at 15 ml/h (250 µl/min) resulting in a combined flow speed of 500 µl/min. Due to the 1:1 addition of the activated PEG solution, the concentration of the magnetic support was diluted to 1 mg/ml during this step. To reduce hydrolysis of the Succimidyl carbonate activated mPEG prior to use, the solution was kept on ice and the reciprocal syringe volume was modified to only hold a maximum of 2 ml[3]. The exact process time during this step was approximately 4.8 minutes.

Process Termination: The PEGylation reaction was stopped by the addition of 50 mM lysine administrated using a reciprocal pump set at 30 ml/h (500 µl/min) through a SHM-type 3A unit resulting in a combined flowrate 1 ml/min after merger with the flow from the PEGylation step. As the volume of the SHM unit used was 0.5 ml the process time was approximately 30 seconds and the resulting particle concentration was 0.5 mg/ml throughout the termination process.

Separation and elution: Unlike the previously described elements, this part of the process was conducted in a batch-wise mode. After leaving the continuous part of the process the magnetic support was led to the HGMS system and collected. After the initial loading of the system, washing buffer (PEG4 buffer) was pumped through for 5 minutes at 20 mg/ml before the system was sealed off enabling the suspension to be pumped around in the loop. The HGMS system was then switched off, the pump speed increased to 50 ml/min and the flow reversed, resulting in the release and circulation of the magnetic support. After circulating the contents of the loop for approximately 5 minutes,

[3] The reciprocal pump was operated at room temperature.

the pump speed was decreased to 20 ml/min, the pump direction reversed and the HGMS system switched on. This process was allowed to proceed for 10 minutes resulting in collection of the support in the magnetic filter. The washing procedure was repeated three times. The valves were subsequently set to bypass the magnetic filter and the system was flooded with elution buffer (100 mM glycine, pH 2.6 supplemented with 5 mM $CaCl_2$) for approximately 5 minutes (pump speed 20 ml/min). The pump was switch off and the valves were readjusted to include the magnetic filter within the closed loop. The flow was reversed and the pump was turned on at maximum speed (~ 60 ml/min) and the flow was recirculated for 10 minutes to achieve elution of the PEGylated conjugates. The HGMS system was turned on, the flow was reversed and the magnetic support was captured as previously described. During the final step the liquid was forced out of the system and collected by filling the system loop with air. A schematic presentation of the separation/elution procedure is available in appendix A.

3.2.7 Analytical methods

BCA-assay: The concentration of total protein was determined using a BCA-assay kit (Pierce, Rockford, Ill, USA) and the method described by Smith et al. (1985). A Cobas Mira Plus robotic spectrophotometer (Roche Diagnostic System, Rotkreuz, Switzerland) with adsorbance measurements at 550 nm was used for the analysis. For each analysis a standard curve was prepared from known concentrations of trypsin and used to determine the protein content of the unknown samples.

Dry weight: The mass of the adsorbents used during the studies was determined after desalting. Desalting was achieved by simply washing the supports at least 3 times with MilliQ water. The particle suspension was then transferred to pre-dried and pre-weighed glass tubes and subsequently dried in an oven at 95°C to constant weight. The mass of particles was then determined from the difference in weight of the tube.

MALDI-TOF mass spectrometry: MALDI-TOF mass spectrometry was performed using a Voyager-DE STR Biospectrometry Workstation (Applied Biosystems, 5791 Van Allen Way, Carlsbad, California 92008, USA). The standard procedure for all the MALDI-TOF measurements performed included a preliminary sample clean up step using 0.6 µl ZipTip-C18 pipette tips (ZTC18S960, Milipore, Billerica, Massachusetts, USA). Prior to the sample clean-up procedure the ZipTip-C18 was pretreated by washing it 5 times in 100% acetonitrile (ACN), 5 times with 1:1 ACN: 0.1% trifluoroacetic acid (TFA) and 5 times in 0.1% TFA. 10 µl of sample was passed through the tip 10 times to allow binding followed by 3 washing steps using 0.1% TFA. The cleaned sample was finally eluted in 1 µl 100% ACN directly on the plate matrix and immediately mixed with 1 µl of 10 mg/ml sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) dissolved in 0.1% TFA solution. The mixed droplets were dried using a conventional hairdryer at low speed. Linear positive spectra were obtained for proteins with sizes between 10 to 40 kDa with a focus set at 24 kDa using 23 KV and a 500 ns delay. All presented spectra were the result of at least 250 overlays and thus 250 shots with the laser. Handling and visualization of MALDI-TOF spectra were achieved using the Voyager control software.

SDS-PAGE: Preparation of protein samples for reducing SDS-PAGE was conducted by addition of 5% v/v mercaptoethanol and 1/4 volume 4x NuPAGE LDS sample buffer (Invitrogen, Carlsbad, California, USA) followed by 10 minutes of heating at 96°C. Reducing SDS-PAGE was performed using the NuPAGE Novex Bis-Tris precast gel system from Invitrogen combined with MES SDS running buffer according to the manufactures reccomendations. The protein bands were visualised with Coomassie blue staining. The SDS-PAGE gel was incubated with shaking for 15 minutes in staining solution (0.35 g/l CRR R-250, 45% v/v Ethanol, 9% v/v Glacial acetic acid) after being pre heated to the brink of boiling in a microwave oven. Background color removal was performed by incubating for 60 minutes in microwave oven pre-heated destaining solution (10% v/v 2-propanol, 10% v/v Glacial acetic acid), or until the protein bands were visible. Gels were then incubated overnight in distilled water with shaking. Gel images were captured using a flatbed scanner (D660U Canon Inc., Tokyo, Japan). Precast SDS-PAGE gels were obtained from Invitrogen (Carlsbad, California, USA).

Trypsin activity: Measurement of enzyme activity toward the small substrate N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) was based on the method of Erlanger et al. (1961). The reaction solution was prepared by combining 7 volumes of 100 mM Tris-HCl (pH 7.5) and 1 volume of a freshly prepared 40 mM BAPNA solution dissolved in DMSO. The assay was performed at 37°C using a Cobas Mira Plus robot spectrophotometer and initiated by adding sample to the reaction mixture. The change in absorbance at 405 nm was recorded and used to calculate the trypsin activity. The units of activity denoted as U_{BAPNA}, are defined as the amount needed to convert 1 μmol BAPNA per second at 37°C. The activity of trypsin toward a large substrate was determined by using the azocasein method as described by Fujimura and Nakamura (1987) with minor modifications. Two volumes of 5% (w/v) azo-casein dissolved in 50 mM This-HCl (pH 7.5) were mixed with seven volumes of 50 mM This-HCl (pH 7.5). To start the assay one volume of sample was then added and the mixture was then incubated for 30 minutes at 37°C. The reaction was stopped by the addition of ten volumes of a 10% (w/v) Trichloroacetic acid (TCA) solution. The samples were placed on ice for 20 minutes and spun at 13000 rpm for 6 minutes (4°C). Finally one volume of

the supernatant was mixed with four volumes of 1.8 M NaOH. The absorbance at 420 nm was determined using a Novaspec II spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and compared with a standard curve prepared using trypsin with a known activity (determined to be 11.9 U_{BAPNA}/mg). Based on data presented by Petersen (2007) and measurements conducted during the current study, the following correlations were established for the native trypsin used here: 1 U_{AZO} corresponds to 199.70 U_{BAPNA} and 1 U_{BAPNA} equals 11.6 mg trypsin.

Quantitative amino acid analysis: The total protein concentration was also determined using quantitative amino acid analysis. First chemical hydrolysis was performed at 110°C for 16 h using 18.5% HCl and 0.1% phenol. The resulting mixture was then processed on a Biochrom 20 Plus apparatus (Biochrom Ltd., Cambridge, UK). Due to the large amounts of lysine added to terminate the PEGylation reaction and the content of glycine in the elution buffer both amino acids were excluded in the quantitative amino acid analysis.

3.3 Results and discussion

The overall aim of this work was to design and test a complete semi-continuous process for the PEGylation of immobilised trypsin consisting of three serial parts denoted adsorption, PEGylation, and elution/separation. Detailed studies of the individual steps were first carried out to establish the most suitable process parameters before the complete process was assembled. Given that the process was to be demonstrated in small scale, and to ensure as much of the process as possible was continuous, it was necessary to eliminate batch reactions whilst still ensuring good mixing of the solutions employed, which contained magnetic particles as sell as viscous PEG solution. This task is not trivial at the small scales chosen here, in which liquids behave differently (e.g. due to low Reynolds numbers) as compared to when larger volumes are employed. Staggered Herringbone Mixers (SHM) were thus chosen to provide a means of continuously mixing the solutions employed.

3.3.1 Investigation of parameters controlling adsorption

One of the most important factors affecting adsorption of trypsin to magnetic supports is the buffer used. However, this choice is further complicated by the fact that the total process should approach being continuous and thus that the solution resulting from the adsorption step will pass directly to the PEGylation step, and then to the HGMF process. Buffering capacity around pH 7-9 is required to promote binding of trypsin to the benzamidine derivatized magnetic support and to ensure efficient coupling of the succimidyl-carbonate monomethoxy polyethylene glycol to lysine residues. Furthermore, the buffer must not contain primary amines (or other reactive groups) in order to avoid undesirable reactions of the activated PEG with the buffer components. This latter requirement rules out more commonly used buffers like tris, HEPES or PIPES.

The influence of buffer composition on adsorption

In previous studies of HGMF by Heebøll-Nielsen (2002) and Gomes (2006) tris-based buffers were used for the adsorption of trypsin to benzamidine coated magnetic supports (100 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$), which are not compatible with the PEGylation chemistry. As an alternative, a borax based buffer with pH 8, designated PEGylation buffer 1 (PEG1 buffer), was employed by Petersen (2007) in the study of adsorption and PEGylation of trypsin in batch reactions. As calcium is known to prevent the problem associated with autoproteolytic activity of trypsin (Sipos and Merkel, 1970), Petersen also introduced buffers containing calcium. However, in the current work, testing of similar buffers during simple flow tests using a prototype SHM-type unit (SHM-type 1A) revealed that the magnetic support formed aggregates, which caused clogging and flow disruption. It was speculated that the buffer composition was the cause of the magnetic supports' behaviour, since aggregation was not seen in other buffers. To circumvent the adsorbent aggregation and clogging problem observed, a modified borax-buffer, denoted PEG4 buffer (100 mM $Na_2B_4O_7$ pH 8, 5 mM $CaCl_2$), containing decreased amounts of salts was used. Initial flow testing using the SHM-type 1A unit and 1 mm Teflon-tube with 6 mg/ml particles at 250 µl/min indicated that the previously observed aggregation behaviour was minimized and clogging was eliminated. Furthermore, when a simple test was conducted in which trypsin was incubated for 3 h at room temperature in PEG4 buffer, no degradation products were seen by SDS-PAGE analysis (Figure 3.5), which confirmed that the lowered calcium concentration was not detrimental to trypsin stability. To evaluate the suitability of the PEG4 buffer during adsorption of trypsin to the magnetic supports, a series of small scale microtube based binding studies were performed.

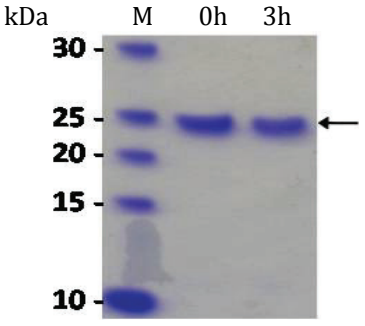

Figure 3.5: Unreduced SDS-PAGE analysis of autoproteolytic activity of trypsin dissolved in PEGylation 4 buffer (PEG4 buffer). The 3h sample was incubated at ambient temperature. 'Oh' represents a control sample (no incubation). The arrow indicates the band corresponding to native trypsin

In order to compare the results with previously published data, additional binding studies were also conducted using the PEG1 buffer employed by Petersen (2007) and the original tris-based buffer utilized by Heebøll-Nielsen (2002) and Gomes (2006).

The results presented in Figure 3.6 illustrate excellent adsorption isotherms in all buffers and all could be fitted well with the Langmuir model. All isotherms had a steep initial slope followed by a plateau, which is consistent with a strong affinity ligand and a single binding site. There were only minor differences in the dissociation constant (k_d) observed when binding was conducted in the different buffers, when either total protein or enzyme activity were used for the isotherms, 4.5 – 5.5 µM (0.11-0.13 mg/ml) or 2.1 – 4.0 U_{BAPNA} /l, respectively (Table 3.2). Furthermore, in all cases the maximum binding capacities (Q_{max}) were calculated to be approximately 200 mg/g or around 8 U_{BAPNA}/g (Table 3.2). The Q_{max}/k_d values for all buffers used, and from both protein and activity measurements, gave very similar results: Between 1.6 and 1.9 l/g and 2.1 – 3.2 l/g, respectively (Table 3.2).

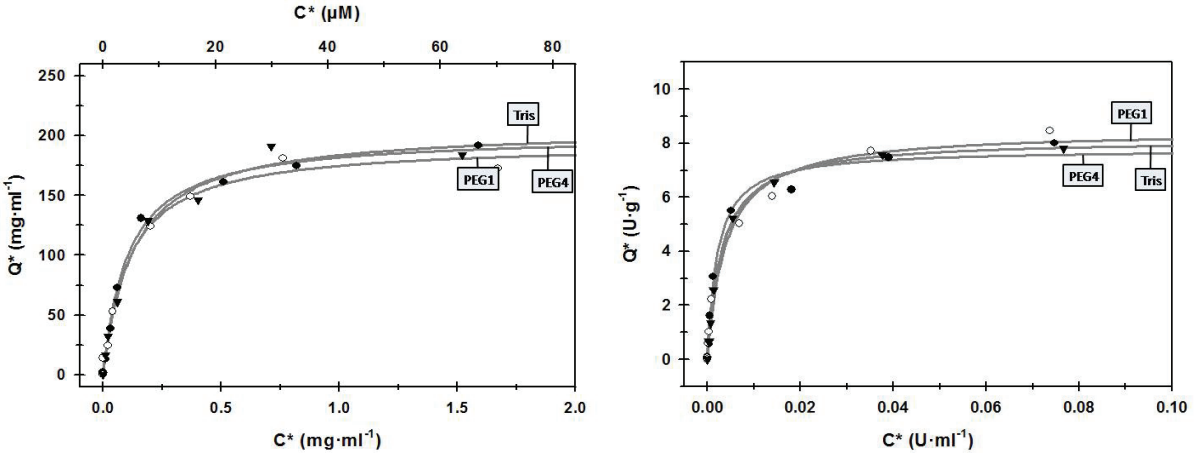

Figure 3.6: Effect of buffer composition on the adsorption of trypsin onto non-porous benzamidine derivatized magnetic supports. *Left:* Adsorption isotherm determined by measuring total protein. *Right:* Isotherm prepared using data obtained from activity measurements. *Both:* (▼) Tris-buffer (100 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$), (●) PEG1 buffer: (200 mM Sodium tetraborate, pH 8.0) and (○): PEG4 buffer (100 mM tetraborate decahydrate, pH 8.0, 5 mM $CaCl_2$). In all cases isotherms were performed as small scale microtube adsorption experiments at room temperature using a 30 minute incubation period. C* represents the concentration of trypsin measured in the supernatant at equilibrium while Q* is the calculated amount of enzyme bound to the support at equilibrium. The data points are fitted to the Langmuir equation using the non-linear least square fitting method (Sigma-plot). The Langmuir parameters are listed in Table 3.2.

Table 3.2: Langmuir model parameters calculated for adsorption isotherms shown in Figure 3.6 for trypsin binding to non-porous benzamidine derivatized magnetic supports. Q_{max} represents the maximum binding capacity while k_d indicates the dissociation value (concentration at which 50% capacity is achieved). Q_{Max}/k_d reflects the initial slope of the isotherm and is referred to as tightness of binding. The Langmuir parameters were determined using both total protein (BCA assay) as well as the activity (BAPNA assay) measurements.

Buffer type	Q_{max}	k_d		Q_{max}/k_d	Q_{max}	k_d	Q_{max}/k_d
	mg/g (Std_{Err})	mg/ml (Std_{Err})	µM (Std_{Err})	l/g	U_{BAPNA}/g (Std_{Err})	U_{BAPNA}/l (Std_{Err})	l/g
PEG4	201.1 (4.74)	0.1055 (0.01)	4.43 (0.42)	1.9	7.79 (0.25)	2.1 (0.3)	3.2
PEG1	194.3 (7.72)	0.1104 (0.02)	4.64 (0.78)	1.8	8.48 (0.45)	4.0 (1.0)	2.1
Tris	207.0 (8.52)	0.1269 (0.02)	5.33 (0.83)	1.6	8.16 (0.17)	3.2 (0.5)	2.6
	Total protein measurements				Trypsin activity (BAPNA)		

The maximum binding capacities (Q_{max}) found here are consistent with those reported by Heebøll-Nielsen (2002), Gomes (2006) and Petersen (2007), who all reported values between 178 and 244 mg/g using a mono-component system. The values for the dissociation constant (k_d) found in the current work are mid-way between those reported by others: Heebøll-Nielsen (2002) and Gomes (2005) both reported values below 1 µM while Petersen (2007) found the value to be above 13 µM. When using activity measurements, values for Q_{max} of 8.61 U/g and k_d of 4.6 U/l were reported by Petersen (2007), which are very similar to those found in the current work (7.8-8.5 U/g and 2.1-4.0 U/l, respectively). Not surprisingly tightness of binding (Q_{max}/k_d) was found to be very different by previous investigators since this parameter depends on both the calculated maximum binding capacity (Q_{max}) and dissociation constant (k_d). According to Franzreb et al. (2006) Q_{Max} should be ≥100 mg/g and tightness of binding should preferably be above 5 g/l. Heebøll-Nielsen (2002) and Gomes (2006) both listed Q_{max}/k_d values above 30 l/g, which on average, are almost 15 times greater than seen in this study, and those of Petersen (2007) (0.8-1.9 l/g). A reasonable explanation for the differences seen among the investigators is presented by Petersen (2007) who points to the reference protein used in the BCA assay as a possible source of the deviations. Both Heebøll-Nielsen and Gomes used protein concentrations expressed as BSA-equivalents whereas in the current work, and that of Petersen (2007), trypsin was used to create standard curves for protein analysis with the BCA assay. Petersen found the correlation between similar concentrations of trypsin and BSA to be 3:2 (trypsin:BSA) when using

the BCA assay Another possible explanation proposed by Petersen (2007) suggests that the choice of buffer and the presence of calcium could play a role, however the data presented in the current study suggests that these effects are of less importance. Finally it should also be pointed out that even though the support was prepared using similar protocols by all parties, it is likely that minor variations in the equipment, batch-size and general approach could result in slightly altered properties of the support and thus altered adsorption profiles.

The effect of temperature on adsorption

For examination of the PEGylation of trypsin immobilised to magnetic adsorbents, the ability to use different temperatures during the PEGylation reaction would be very useful. However, it is important that the trypsin does not disassociate from the supports in the temperature ranges to be employed. Batch binding studies were therefore conducted at temperatures ranging from 12°C to 45°C using small scale microtube based batch binding studies. The temperature range included in the study was mainly based on the observation that insoluble precipitates would form rapidly under 10°C, presumed to be the result of the formation of calcium salts, and a limitation imposed by the stability of SC-mPEG, which is hydrolyzed by water with increasing rate as the temperature is raised.

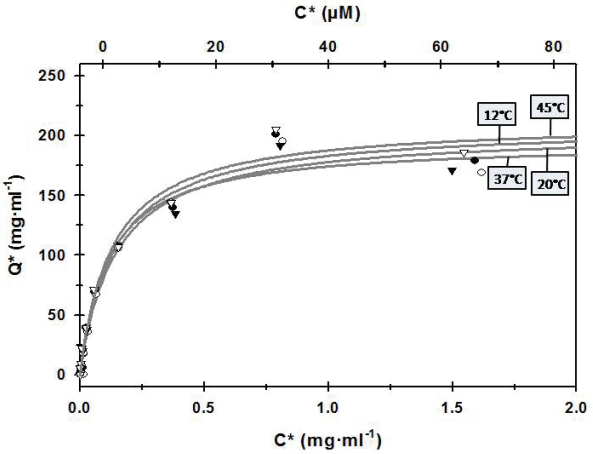

Figure 3.7: The impact of temperature on adsorption of trypsin by benzamidine linked magnetic supports. The isotherms were obtained in small scale batch binding studies at 12°C (●), 20°C (○), 33°C (▼) and 45° (▽) using a 30 minute binding period then measurements of unbound protein in the supernatant. The trypsin used was dissolved in PEG4 buffer. C* represents the concentration measured in the supernatant at equilibrium while Q* is the calculated amount of protein bound to the support at equilibrium. The data points are fitted to the Langmuir equation using non-linear least square function. The Langmuir parameters are presented in Table 3.3.

The results in Figure 3.7 and Table 3.3 show there were no significant differences in the maximum binding capacity (Q_{max}) or dissociation constants (k_d) found as temperature was raised from 12°C to 45°C and the values were comparable with the values found earlier of 200 mg/g and 5-6 µM, respectively (Table 3.2). Tightness of binding was found to be essentially identical with the earlier values (Table 3.2). It was therefore concluded that temperatures between 12°C and 45°C could be examined as a means of controlling PEGylation without affecting adsorption.

Table 3.3: Langmuir binding parameters for the isotherms shown in figure 3.7. Q_{max} represents the maximum binding capacity while k_d indicates the dissociation value (concentration at which 50% capacity is achieved). Q_{Max}/k_d reflects the initial slope of the isotherm and is referred to as tightness of the binding.

Temperature °C	Q_{max}	k_d		Q_{max}/k_d
	mg/g (Std_{Err})	mg/ml (Std_{Err})	µM (Std_{Err})	l/g
12	208.6 (11.8)	0.1389 (0.028)	5.84 (1.2)	1.5
20	203.8 (12.3)	0.1457 (0.030)	6.12 (1.3)	1.4
37	194.8 (9.53)	0.1176 (0.022)	4.94 (0.90)	1.7
45	212.2 (11.4)	0.1303 (0.026)	5.47 (1.1)	1.6
	Total protein measurements			

Kinetics of adsorption of trypsin by benzamidine linked supports

Adsorption of molecules on to non-porous magnetic supports is generally considered to be a fast process (Franzreb et al., 2006), however to confirm this for the system under study here, a kinetic batch binding study was performed. The investigation was carried out at room temperature using various incubation periods from 60s to 0.5 h and the unbound protein fractions were analyzed for both total protein and enzyme activity, using BCA and the BAPNA based activity assay.

As expected, the investigation revealed very fast adsorption kinetics for the trypsin/benzamidine model system and regardless of the incubation period, the binding isotherm obtained could be fitted with the Langmuir model. After a one minute incubation period the bound protein fraction had reached approximately 70-75% of the highest binding capacity (Q_{max}) at high protein loads (Table 3.4). However an interesting change was observed for the apparent dissociation constant (k_d) which had almost doubled from 5.8 to 9.8 µM (0.14 - 0.23 mg/ml) or 0.7 to 1.4 U/l when the time for

adsorption was reduced from 0.5 h to 60 s. As a result, the tightness of binding decreased almost three fold from 1.5 L/g to 0.6 L/g (when total protein was measured) and 10.8 to 3.8 l/g in the case of activity measurements with BAPNA when adsorption time was reduced to 60 s.

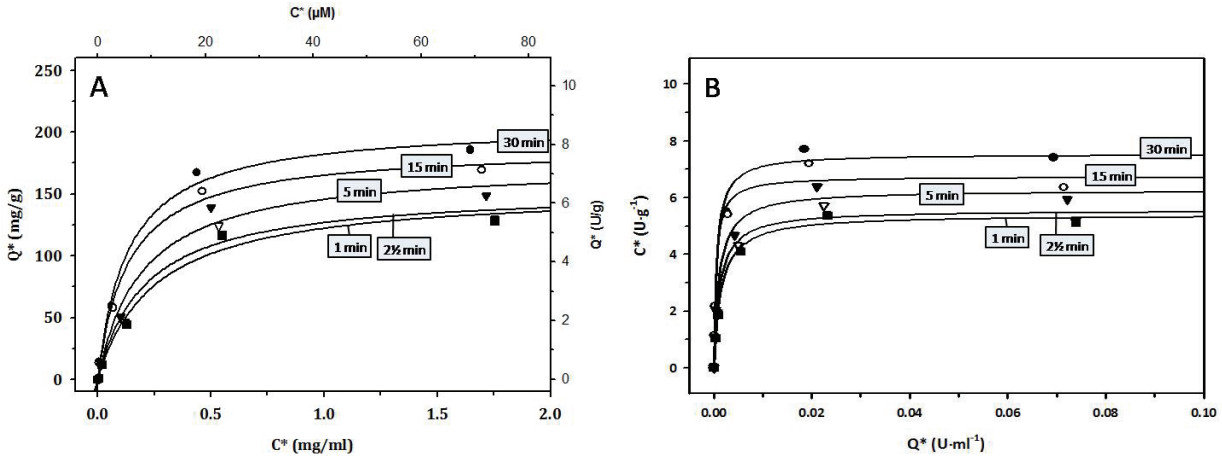

Figure 3.8: Kinetic study of adsorption of trypsin on to non-porous magnetic supports derivatized with benzamidine. (A) Isotherm prepared from total protein measurements. (B) Isotherm prepared from activity measurements. A+B**:** The isotherms were obtained in small scale studies at room temperature using PEG2 buffer at incubation periods of 1 minute (■), 2.5 minutes (∇), 5 minutes (▼), 15 minutes (○) and 30 minutes (●). C* represents the concentration measured in the supernatant at equilibrium while Q* is the calculated amount of protein bound to the support at equilibrium.

Table 3.4: Langmuir model parameters for isotherms of the kinetics of trypsin adsorption presented in Figure 3.8. Q_{max} represents the maximum binding capacity while k_d indicates the dissociation value (concentration at which 50% capacity is achieved). Q_{Max}/k_d reflects the initial slope of the isotherm and is referred to as tightness of the binding.

Time minutes	Q_{max}	k_d		Q_{max}/k_d	Q_{max}	k_d	Q_{max}/k_d
	mg/g (Std_{Err})	mg/ml (Std_{Err})	µM (Std_{Err})	l/g	U_{BAPNA}/g (Std_{Err})	U_{BAPNA}/l (Std_{Err})	l/g
30	204.6 (6.07)	0.1376 (0.017)	5.78 (0.73)	1.5	7.53 (0.285)	0.6 (0.1)	12.6
15	187.5 (7.07)	0.1333 (0.022)	5.60 (0.90)	1.4	6.74 (0.282)	0.5 (0.1)	13.5
5	174.1 (12.9)	0.1947 (0.054)	8.18 (2.29)	0.89	6.26 (0.223)	0.9 (0.2)	5.69
2.5	152.7 (11.6)	0.2131 (0.059)	8.95 (2.47)	0.72	5.56 (0.229)	1.2 (0.3)	4.63
1	148.4 (13.6)	0.2328 (0.076)	9.78 (3.18)	0.64	5.39 (0.149)	1.5 (0.2)	3.59
	Total protein measurements				Trypsin activity (BAPNA)		

Gomes (2006) also investigated the kinetics of adsorption of trypsin using similar magnetic particles. She reported that approximately 4.5 g/l support was able to remove close to all activity from crude whey containing 0.15 g/l trypsin after only 4 minutes

incubation. This result, even though performed under slightly different conditions in a complex system (crude whey), is consistent with the isotherms obtained using a mono component system presented in the current study. In light of the above results, it was concluded that an incubation time of 5 minutes in the continuous adsorption reactor would be acceptable for loading the magnetic adsorbents.

3.3.2 Investigation of parameters controlling PEGylation

Petersen (2007) demonstrated in a proof-of-concept study that it was possible to PEGylate immobilized trypsin in a batch-wise microtube based process using 5 kDa succimidyl carbonate monomethoxy polyethylene glycol (SC-mPEG). However, in order to develop an 'online' semi-continuous process, it was necessary to further extend the understanding of parameters controlling the PEGylation reaction.

Study of the effect of reaction time and SC-mPEG concentration on PEGylation of immobilised trypsin

To investigate the influence of SC-mPEG concentration and reaction time on the PEGylation of trypsin immobilized on magnetic supports, a series of reactions were performed using various incubation periods and increasing concentrations of activated mPEG. In each reaction 2 mg/ml benzamidine derivatized magnetic support was preloaded using 2 mg/ml trypsin for 30 minutes giving a loading of ca. 190 mg trypsin/g support. The PEGylation reactions were carried in 1 ml scale by mixing 500 µl preloaded and washed support with 500 µl of the of SC-mPEG solution in question, then incubating for the desired time, capturing the supports magnetically, washing, eluting and analysing the samples by SDS-PAGE. Figure 3.9 clearly shows that increasing concentrations of SC-mPEG as well as longer reaction times both lead to the appearance of discrete bands with higher molecular weight in a positively correlated manner. At the lowest SC-mPEG concentration, two bands above the molecular weight of native trypsin 23.8 kDa) can be clearly seen and are separated by ca 5 kDa, fitting the theoretical increase in size expected to occur by attachment of a 2 kDa PEG-group. It should however be noted that

PEGylated proteins appear to have much higher molecular weights than expected due to the large hydrodynamic radius of the PEG, which slows movement during SDS-PAGE.

At the highest concentration of SC-mPEG discrete bands are still seen, however they are less intense, hazier, appear more dispersed and conjugates with higher molecular weights are seen. Lanes containing samples originating from the 80 mg/ml SC-mPEG reactions at the longest reaction times, appear to be virtually empty (Figure 3.9). It is believed that PEGylation did take place under these conditions, but that many PEG groups were attached to each protein, which led to less conjugates having only 1-2 PEG molecules attached.

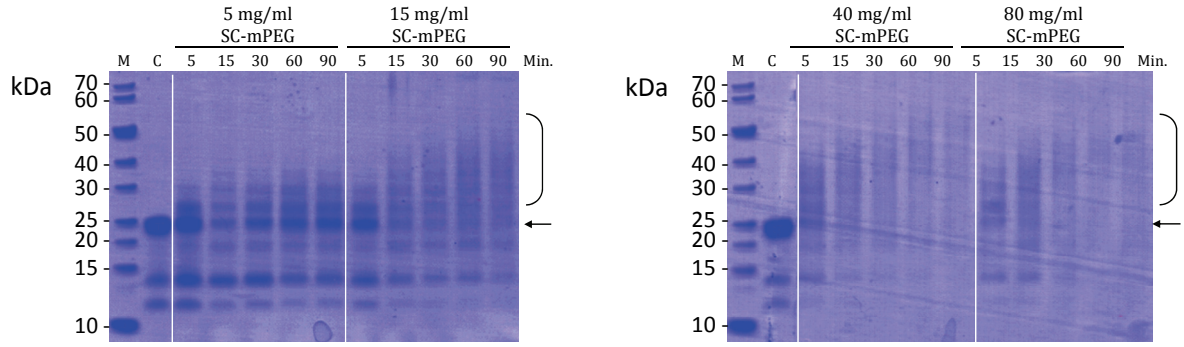

Figure 3.9: Coomassie stained SDS-PAGE gels of trypsin eluted from magnetic supports after PEGylation. The effect of variable concentration of 2 kDa SC-mPEG and reaction time on the PEGylation of immobilized trypsin on benzamidine derivatized magnetic particles is shown. The individual reaction times and SC-mPEG concentrations used are indicated at the top of the gels. C referrers to a control sample 'PEGylated' using 0 mg/ml SC-mPEG. The arrow indicates the native trypsin band and the brackets indicate the position of the PEGylated trypsin conjugates.

Heavily PEG-derivatised proteins are known to be shielded by the PEG groups (Roberts et al., 2002), leading to poor dye staining and thus reduced visualisation on Coomasie stained SDS-PAGE gels. Similar observations were described by Petersen (2007), who performed PEGylation of trypsin using 5 kDa SC-mPEG, and overcame the problem by employing a specialized iodine staining technique to improve the detection of high molecular weight conjugates. However, despite attempts to utilize similar methods in this study, no improvement of the visualization was seen, which is presumed to be due to the smaller sized PEG used in this study (*i.e.* 2 kDa).

Another important factor which may contribute to decreased intensity of the bands is the dispersed nature of the PEG (see figure 1.3), which gives rise to populations of conjugates with less discrete molecular weights and thus less defined bands. The results

suggest strongly that addition of defined numbers of PEG groups to immobilized trypsin can be controlled by varying either PEG concentrations and/or reaction time. However, to confirm that the systematic increase in molecular weight of the bands seen by SDS-PAGE corresponded to the progressive addition of one PEG group, the technique of Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) was used.

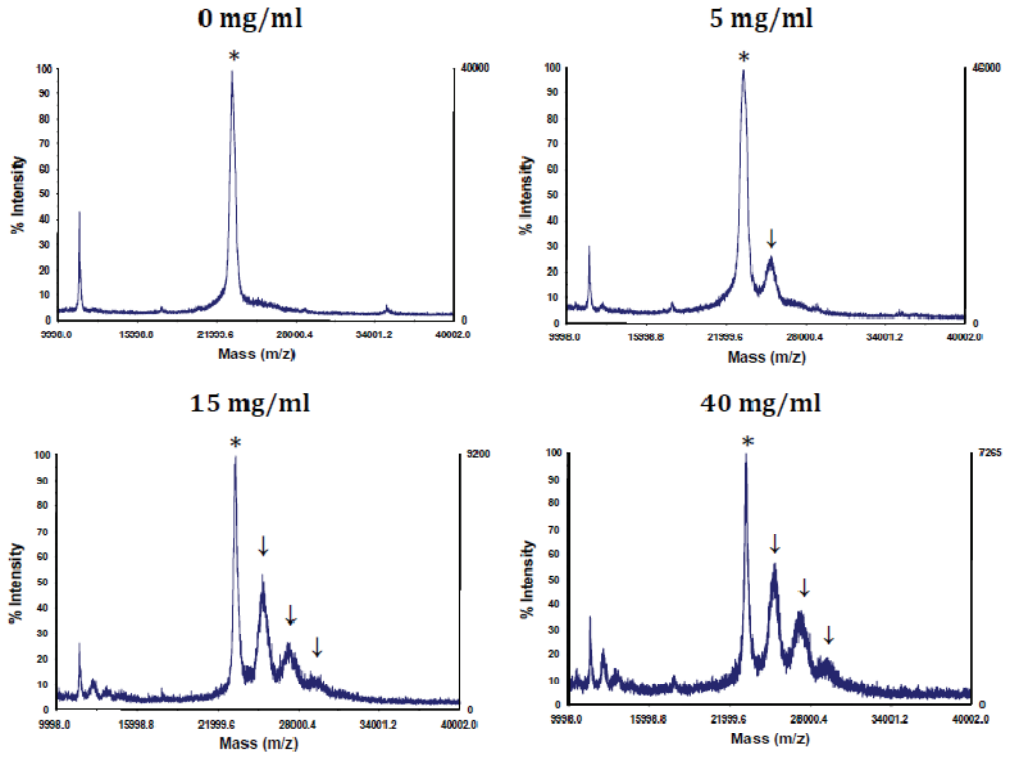

Figure 3.10: MALDI-TOF spectra showing the results of a 5 minute PEGylation reaction of immobilized trypsin using various concentration of 2 kDa SC-mPEG. (*) indicates the native trypsin peak while arrows show the position of peaks corresponding to PEG conjugates.

Similar samples analysed by SDS-PAGE in Figure 3.9 were analysed using MALDI-TOF and the spectra presented in Figure 3.10, show the result of a 5 minute PEGylation reaction carried out using various concentration of SC-mPEG. The results confirm what was observed from the SDS-PAGE gels shown in Figure 3.9. As the concentration of activated mPEG was increased, peaks with higher mass per charge (m/z) started to appear, which were separated by approximately 2 kDa, corresponding to the average mass of a single attached PEG molecule. It can furthermore be observed that the peaks with higher molecular mass than native trypsin show lower intensity but a broadened

signal. The spectra obtained for the reaction using 5 mg/ml SC-mPEG show two peaks corresponding to native trypsin and a single group of positional PEG isomers carrying one covalently attached PEG group. The reactions performed using 15 and 40 mg/ml activated PEG showed multiple peaks, which besides native trypsin, represented several groups of positional isomers with one, two and three PEG-groups attached.

MALDI-TOF analysis was also employed to extend the investigation of the effects of reaction time on the PEGylation process, and the results were also found to be in full agreement with the previously observed results seen in Figure 3.8. PEGylation reactions were conducted with a fixed concentration of 10 mg/ml 2 kDa SC-mPEG and samples were taken after 1, 2, 5, 15, 30, 60 and 120 minutes. As can be seen in Figure 3.11 the formation of conjugates can already be detected after 60 s. Three peaks are visible corresponding to native trypsin and two groups of positional isomers carrying either one or two PEG groups. After a 5 minute reaction period, the two PEG-conjugate peaks showed an increased intensity indicating that higher amounts were formed. Furthermore, an additional signal at even higher mass/charge was seen indicating formation of positional isomers with three PEG groups attached. The spectrum of the 15 minute PEGylation sample shows a further increase in the intensity of the conjugate dependent peaks and the observed trend continued as the time frame of the reaction was extended up until 60 minutes, where as many as four peaks each corresponding to separate group of positional isomeric species can be seen. By comparing the 60 and 120 minute reactions it can be concluded that no further increase in the number of peaks occurs, suggesting that attachment of four PEG groups to trypsin represents the maximal achievable number under immobilized conditions (Figure 3.11). Instead, the intensity of the peaks originating from the PEG-conjugates formed continued to increase, while the signal for native trypsin appears to be less intense compared to the peak corresponding to conjugates with a single PEG group attached[4]. The results were found to be in agreement with a similar investigation by Petersen (2007) based on the use of 5 kDa SC-

[4] It should be noted that MALDI-TOF is generally not considered a quantitative method but rather a qualitative tool. Thus the intensity, or area under the peaks, cannot be used as an exact measure of the amount of a substance but rather as an indication of the trend. It should also be pointed out that the decreased signals observed to the far left of most of the spectra are caused by measurements of proteins carrying a double charge rather than the more commonly observed single charge. These signals should therefore be considered echoes and not be interpretated as results.

mPEG, which showed a similar positive correlation between the reaction time and the number of positional isomeric species formed.

To determine if there was a difference between PEGylation of immobilised trypsin compared to trypsin free in solution, a series of PEGylation reactions were performed without the use of magnetic support. A solution of 0.4 mg/ml trypsin was mixed with 10 mg/ml SC-mPEG and samples were collected after 5, 15 and 60 minutes, and were immediately subjected to purification using C-18 packed Ziptips as described under materials and methods, and then analyzed using MALDI-TOF.

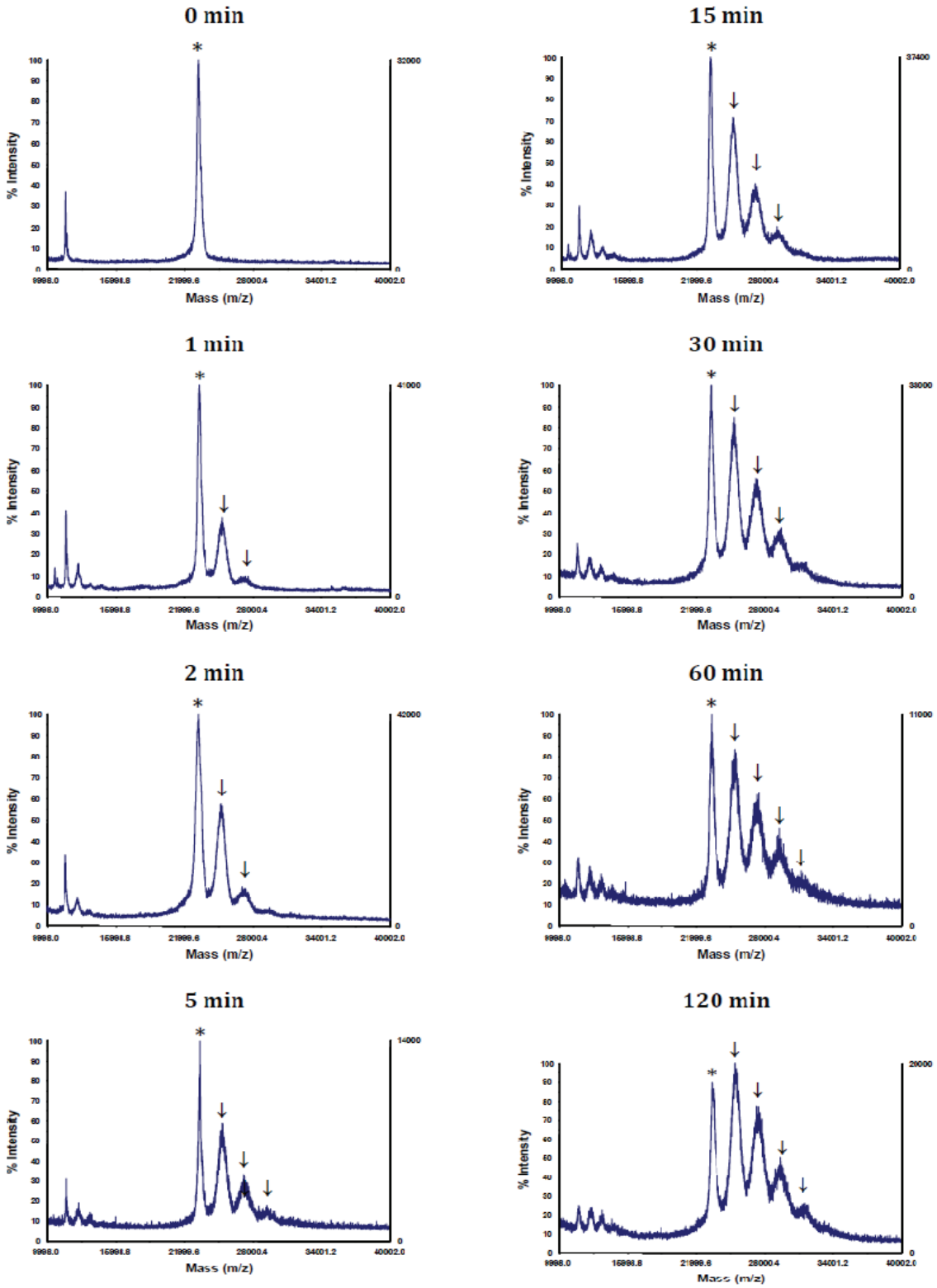

Figure 3.11: MALDI-TOF spectra showing PEGylation of immobilized trypsin with 10 mg/ml SC-mPEG at various time points. (*) indicates the native trypsin peak while arrows show the position of peaks corresponding to PEG conjugates.

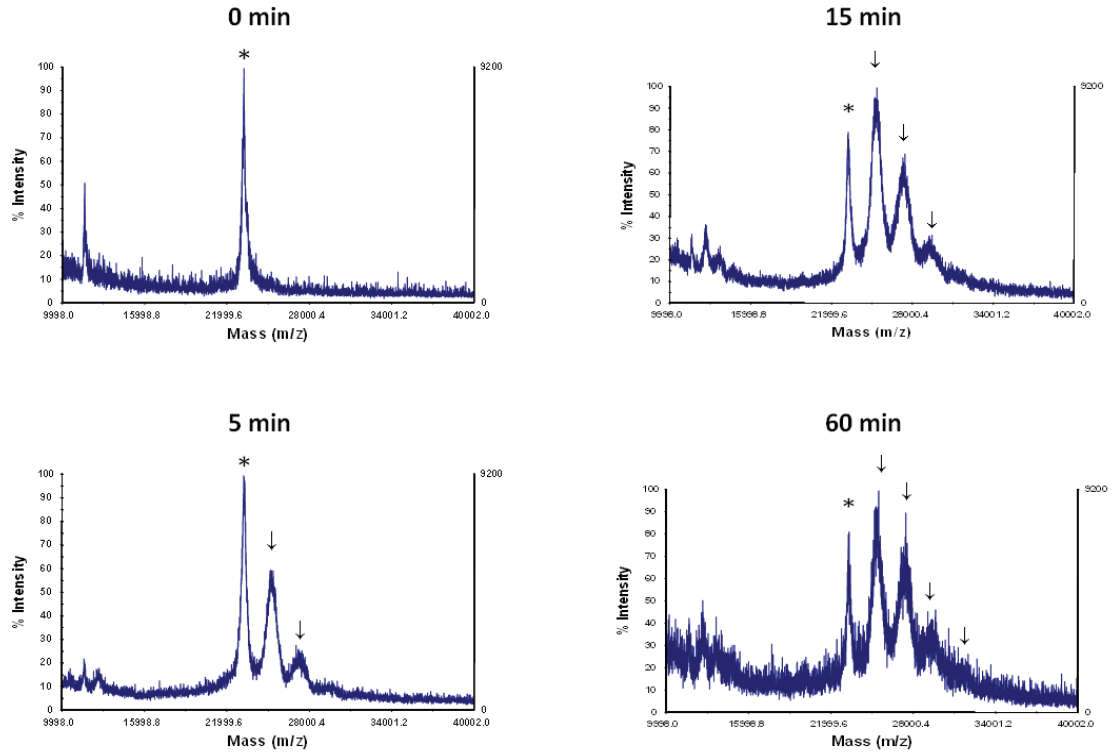

Figure 3.12: MALDI-TOF spectras showing the results of PEGylations of trypsin in free solution using 10 mg/ml 2 kDa SC-mPEG at various time points. (*) indicates the native trypsin peak while arrows shows the position of peaks corresponding to PEG conjugates.

As expected, the results presented in Figure 3.12 showed a positive correlation between reaction time and the degree of PEGylation obtained. However it can be seen that the rate of PEGylation is faster in free solution than for immobilised trypsin. Comparison of the 5 and 15 minute reactions for immobilised trypsin in Figure 3.11 with the corresponding reactions in 3.11 indicate that the two 5 minute reactions appear to have a very similar profile, with a large native trypsin peak and 2-3 smaller peaks corresponding to the conjugates formed. However, in the MALDI-TOF spectrum originating from the analysis of the 15 minute reaction with immobilized trypsin, the native peak remains the most intense, whilst for the case with free trypsin, the highest intensity was found for the conjugate carrying a single PEG group. This observation implied that even though the PEGylation reaction initially occurred at similar rates it subsequently slowed down for the immobilized trypsin in comparison to free trypsin, perhaps due to steric hindrance. When the spectrum originating from the 120 minute reaction with immobilized trypsin (Figure 3.11) is compared to that from the 60 minute

reaction with free trypsin the same spectra and ratios between native trypsin and the PEG-conjugate are seen indicating that both reactions progressed to a similar final stage.

Effects of reaction temperature on the formation of PEG conjugates

Investigation of the effects of temperature on the adsorption of trypsin by benzamidine linked magnetic support showed that this parameter had no detectable influence on this step. This result permitted studies to be conducted to determine the extent by which the PEGylation reaction could be controlled by varying temperature. Two independent series of reactions were carried out using various concentration of SC-mPEG at different temperatures. The first series of PEGylations were carried out using a reaction time of 30 minutes, in tubes placed in a polymerase chain reaction hybridization oven (Hybaid Maxi-14, Thermo Fisher Scientific, 81 Wyman Street, Waltham, MA 02454, USA) with rotary action to ensure good mixing and temperature control. Different concentrations of activated mPEG and temperatures ranging from 20-45°C were used.

The PEGylated samples were analyzed using reduced SDS-PAGE and the observed effect could be divided into two separate groups (Figure 3.13). At 20°C and 30°C no apparent difference could be seen. However as the temperature was elevated further to 37°C the formation of conjugates appeared to be reduced. This trend continued when PEGylation was carried out at 45°C and in this case, conjugates were only formed in the reaction using the highest PEG concentration (40 mg/ml SC-mPEG).

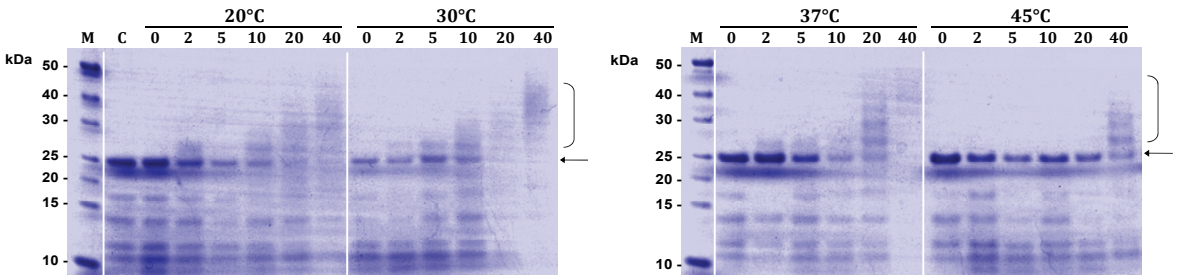

Figure 3.13: Reduced SDS-PAGE gels stained with Coomassie blue showing the effects of temperature on the formation of PEG conjugates when using various concentrations of SC-mPEG. The PEGylation reactions were performed using 30 minute reaction times on trypsin immobilized on magnetic supports. Mixing was performed using a rotating hybridization oven at temperatures (top) and SC-mPEG (below) concentrations are indicated at the top of the gels. The arrow indicates the native trypsin band while the bracket indicates bands corresponding to PEG-conjugates. 'C' indicates an untreated control sample. 'M' designates molecular weight markers.

The results of the investigation of the effects of temperature on the PEGylation reaction were not as expected and to further explore the issue, an additional series of temperature dependent PEGylations were carried out over a wider temperature range (12°C - 45°C) and for a shorter time of 5 minutes. The samples were analyzed using MALDI-TOF and the spectra obtained are shown in Figure 3.14.

The results in Figure 3.14 are consistent with what was seen in Figure 3.13, and clearly illustrated that the highest degree of reactivity, and thus the highest degree of modified trypsin, was found for the reaction carried out at 20°C. For the reactions carried out at 12°C or 33°C conjugates were produced, however the intensity and the number of detectable peaks corresponding to PEGylated positional isomers were decreased as compared to the reactions performed at 20°C using similar SC-mPEG concentration. At 45°C the spectra showed that essentially no conjugates were formed.

The reasons for the observed effects of temperature are believed to be due to a combination of effects on reaction rate and on hydrolysis of the activated PEG. At 12°C the extent of PEGylation is reduced most likely due to temperature dependence of reaction rate, which is slowed down as temperature is decreased. However, at temperatures above 20°C it is presumed that there is an increased rate of hydrolysis of the acyl bond in the SC-mPEG, which leads to an increased rate of inactivation of the reactive PEG molecule (Roberts et al., 2002). When the rate of hydrolysis exceeds the rate of reaction of the PEG with amine groups on the trypsin at elevated temperatures, less PEGylation occurs.

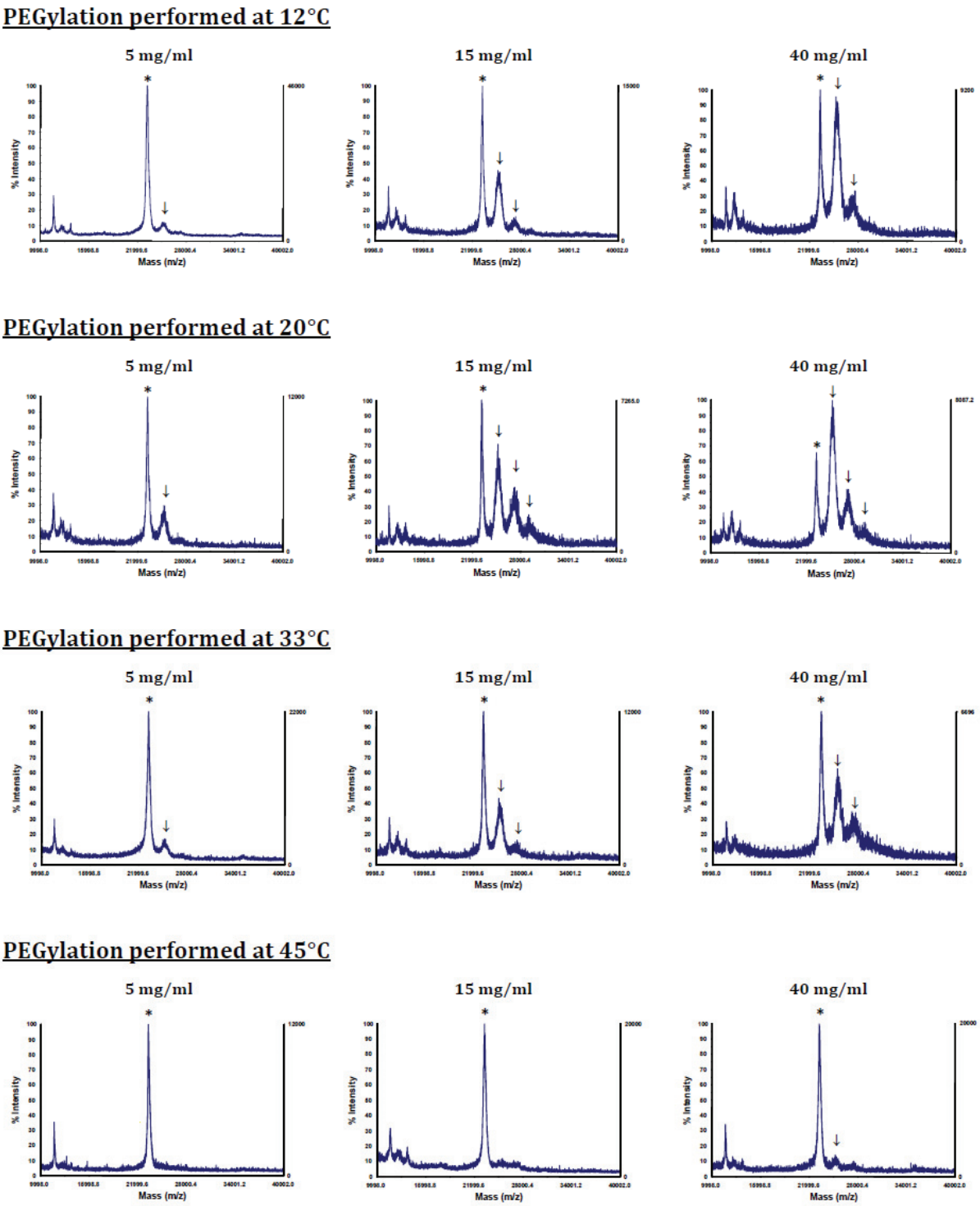

Figure 3.14: MALDI-TOF spectra showing the effects of temperature on the PEGylation of immobilized trypsin: (*) indicates the native trypsin peak while arrows shows the position of peaks corresponding to PEG conjugates. PEGylation reactions were conducted for 5 minutes.

Investigation of methods to terminate the PEGylation reaction

In order to set up a robust, stable and scalable PEGylation process it was necessary to determine how the process could be efficiently terminated, which is a prerequisite for ensuring tight control of the reaction time. The above study of the effects of temperature on the PEGylation process suggests that heating may be a possible method by which the reaction could be stopped. However, pH could potentially also serve as the means of controlling the process since the chemistry of the PEGylation reaction is dependent on the ε-amine of lysine residues being deprotonated. A third alternative may be competition for the activated PEG by adding a source of amine groups to the reaction. For examination of the three possible methods for reaction termination, the ability of each method to inhibit PEGylation of immobilised trypsin, rather than to stop it after it had been started was employed. The immobilised trypsin was subsequently washed and eluted and analysed by SDS-PAGE.

Heat as the means of termination was tested using the following approach. Two mg of trypsin preloaded magnetic supports were suspended in 500 µl PEG4 buffer and subsequently added to a 2x SC-mPEG solution, which prior to use were heated to 45°C for 60 seconds. The results in Figure 3.15 (lane 2 in each gel) show that bands corresponding to PEG-conjugates were produced, demonstrating that this approach did not inhibit PEGylation of the immobilised trypsin. When the results in Figure 3.15 are compared to those in figure 3.13 and 3.14 it can be seen that the heat exposure used prior to the PEGylation reaction did not fully inactivate the SC-mPEG reagent. This observation suggests that longer incubation times than 1 minute would be needed to hydrolyse the unreacted SC-mPEG in the process proposed, before the reaction would stop, making this approach unsuitable.

To terminate the PEGylation process by lowering pH, Petersen (2007) previously used the addition of 5 volumes of 0.01 M HCl which was found to reduce pH below 5. If pH becomes too acidic the succimidyl carbonate activated PEG could react with tyrosine and histidine residues (Roberts et al., 2002). Furthermore, very low pH could also result in desorption of the reversibly bound trypsin (pH 2.6 is used for elution). In light of the above, the investigation of the ability of pH to block the reaction was limited to a decrease from pH 8 to 6.5 by adding 2 mg trypsin preloaded magnetic particles

suspended in a 1:1 mixture of PEG4 buffer and 100 mM acetic acid to the same volume of 2 kDa SC-mPEG solution also suspended in 1:1 PEG4:AcOH. The results in Figure 3.15 (lane 1 in each gel) showed many bands corresponding to PEG conjugates, indicating that this method was not suitable for stopping PEGylation.

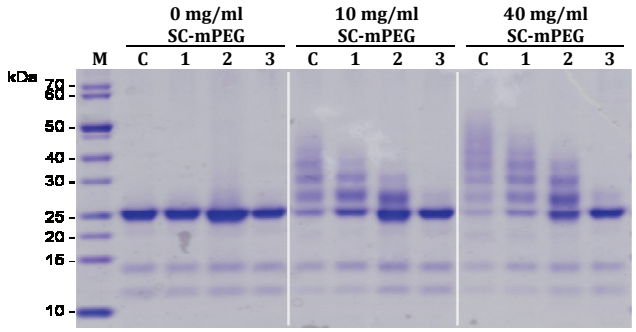

Figure 3.15: Reduced SDS-PAGE gels showing the result of the investigation of the effect of various methods aimed at blocking the PEGylation reaction. (C) Control sample (Not PEGylated), (1) Acetic acid, (2) heating, (3) Lysine. Left: Blocking of PEGylation of immobilized trypsin. Right: Blocking of PEGylation of free trypsin

For the study of lysine as a means of stopping the PEGylation reaction, a similar approach to that used for pH was employed: SC-mPEG in PEG 4 buffer was added to 2 mg of trypsin preload supports suspended in PEG4 buffer supplemented with 70 mM lysine. The final concentrations of SC-mPEG in each of the mixtures were 0 mg/ml, 10 mg/ml or 40 mg/ml and the reactions were allowed to proceed for 30 minutes at room temperature with vigorous shaking. The results in Figure 3.15 (lanes 3 in each gel) shows that only a single weak band corresponding to one PEG conjugate was seen when treated with lysine. To gain further insight into the lysine approach, additional PEGylation reactions were performed with the aim of establishing the concentration of lysine need to terminate the reaction. The reactions were carried out using a 40 mg/ml SC-mPEG concentration and buffers supplemented with variable concentrations of lysine. The eluted samples were analyzed using MALDI-TOF (Figure 3.16). The data show that increasing the concentration of lysine from 0 mM to 25 mM led to a reduction in the numbers of PEG conjugates from four to zero as seen from the MALDI-TOF analysis. It was thus concluded that complete blocking of the reaction was achieved using 25 mM lysine during a PEGylation process started with 40 mg/ml of 2 kDa SC-mPEG. For this reason 25 mM lysine was used to ensure complete cessation of PEGylation (added as 50 mM and diluted 1:1 with the reaction).

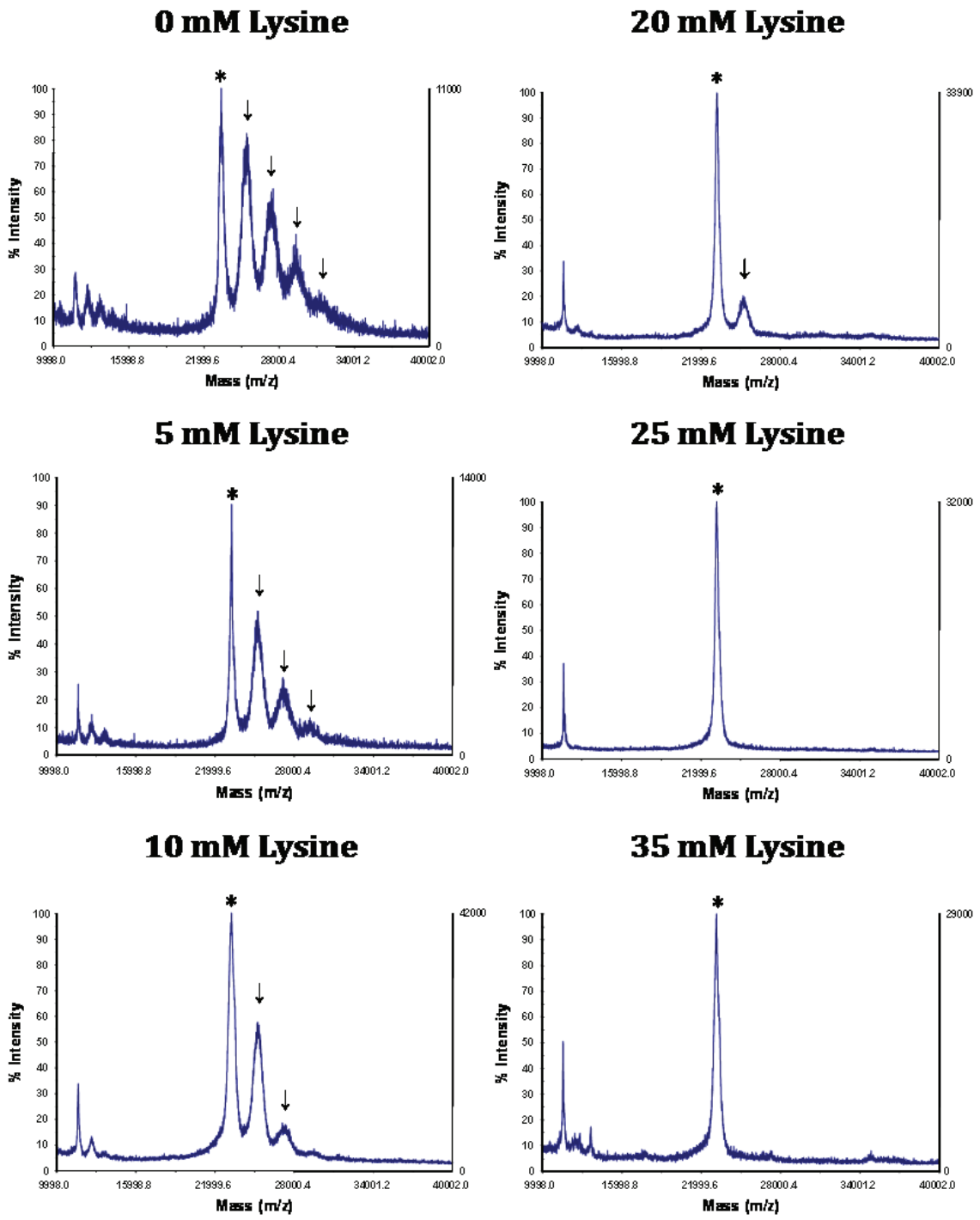

Figure 3.16: MALDI-TOF spectra showing the impact of various concentrations of lysine on a PEGylation reaction carried out on immobilized trypsin. (*) indicates the native trypsin peak while arrows shows the position of peaks corresponding to PEG conjugates. Lysine was present in the solution with pre-loaded magnetic adsorbents before addition of SC-mPEG.

Investigation of the activity of PEGylated conjugates

One of the objectives of immobilising the protein prior to PEGylation is to deliver conjugates which are different to those possible to make in free solution. Immobilisation of trypsin via the active site may protect it during PEGylation delivering more active product than if PEGylation of free trypsin was used. The hypothesis was investigated by Petersen (2007) for trypsin-PEG conjugates derived from batch reactions with 5 kDa SC-mPEG by measuring enzyme activity towards the small substrate BAPNA (435 Da) as well as the larger azocasein (25 kDa). The results of this investigation showed that PEG conjugates prepared using immobilized trypsin displayed increased activity towards both BAPNA and azocasein than unmodified trypsin, while PEGylation performed with trypsin free in solution gave rise to conjugates showing unchanged or decreased activity. The PEG conjugates used in the investigation of the activity by Petersen were prepared from immobilised and free trypsin using similar SC-mPEG concentrations and reaction times, however that study did not take into consideration that conjugates produced with free trypsin had more PEG groups attached than those produced with immobilised trypsin. Thus the differences in activity seen may be a result simply of differences in the degree of PEGylation (the number of attached groups), rather than a difference in placement of the PEG groups arising for example from protection of the active site by immobilisation.

In the current work it was desired to compare the activity of conjugates prepared with trypsin either in free solution or immobilized, and which had the same numbers of covalently attached PEG groups. PEGylation of free and immobilised trypsin was therefore carried out using different concentrations of SC-mPEG for 5 minutes at room temperature. When immobilized trypsin was used, PEG concentrations of 10 mg/ml, 20 mg/ml and 40 mg/ml were employed which yielded conjugates with 1xPEG, 2x PEG and 3x PEG groups attached, respectively. For free trypsin, activated PEG concentrations of 2.5 mg/ml, 5 mg/ml and 10 mg/ml produced conjugates with 1x PEG, 2x PEG, and 3x PEG, respectively. The reactions were in all cases terminated by addition of 1 volume of PEG4 buffer supplemented with 50 mM lysine. Subsequently, for the case of PEGylation of free trypsin, unreacted PEG was removed by diafiltration and washing using cellulose in-tube filters with a molecular weight cut-off of 10 kDa (Millipore Corp., Billerica, MA,

USA) as described by Petersen (2007). The resulting mixtures retained in the filters were analyzed by reduced SDS-PAGE, and samples displaying similar patterns of PEGylation were identified (Figure 3.17). The protein concentration for immobilised and free PEGylated trypsin was determined by quantitative amino acid analysis (see materials and methods). The activity of the selected PEGylated samples were analyzed using BAPNA as well as azocasein, and the specific activities were calculated using the protein concentrations obtained by the quantitative amino acid analysis (Table 3.5).

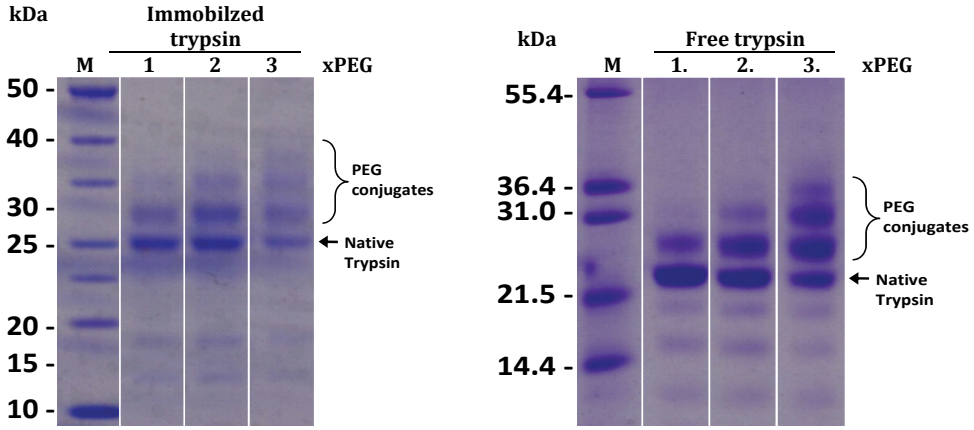

Figure 3.17: Reduced SDS-PAGE showing the samples used to measure the activity of PEG-conjugates. The left gel shows the analyzed samples prepared with immobilized trypsin, while the right gel shows the samples prepared by PEGylation of trypsin free in solution. The maximum number of PEG groups is noted at the top of each lane. 'M' indicates molecular weight standards.

Table 3.5: Specific activities of conjugates towards BAPNA and azocasein. The table lists the measured activities as well as the calculated specific activity. The fold changes shown are normalised to the values obtained for native unPEGylated trypsin, which had been subjected to the same process as the rest of the samples, except that SC-mPEG was not added to the reaction mixture. The 'type' indicates the maximum number of associated PEG groups.

Type	Method	Sc-mPEG	Azocasein		BAPNA		
		mg/ml	$U_{Azocasein}$/g	Fold change	U_{BAPNA}/g	Std_{error}	Fold change
Native	Immobilized	0	2.51	**1.00**	249	6.4	**1.00**
1xPEG	Immobilized	10	2.90	**1.16**	296	4.8	**1.19**
2xPEG	Immobilized	20	3.35	**1.33**	328	7.0	**1.31**
3xPEG	Immobilized	40	4.72	**1.88**	263	49	**1.06**
Native	Free	0	3.24	**1.00**	374	35	**1.00**
1xPEG	Free	2.5	3.88	**1.20**	381	87	**1.02**
2xPEG	Free	5	2.68	**0.83**	252	76	**0.68**
3xPEG	Free	10	2.54	**0.78**	149	7.8	**0.40**

The results in Table 3.5 show that in general, the PEG conjugates produced by immobilisation of the trypsin had greater activity than their counterparts prepared with free trypsin. The conjugates prepared with immobilized trypsin had no loss of activity toward the small substrate BAPNA, and in fact had a slight increase in activity for the first two conjugate types (with one and two PEG groups), followed by a drop back to the native level for conjugates with 3 PEG groups. For the measurements conducted for the corresponding freely PEGylated samples a negatively correlated tendency was seen. As the number of attached PEG groups increased, a clear drop in activity was seen (Table 3.5) ,suggesting that the associated PEG groups interfere with the activity as was seen by Petersen (2007), presumably due to sterical hindrance. Similar trends were seen when activity towards the larger substrate azocasein was examined. For the samples prepared with immobilized trypsin a positively correlated tendency was seen between the number of covalently attached PEG groups and activity. In fact for the sample with the highest degree of PEGylation, the specific activity was elevated by almost 90%. In contrast the opposite trend can be observed for the freely PEGylated samples. Even though the activity for the sample with the conjugates with one attached PEG group increased by approximately 20%, conjugates with two or three PEG groups showed a clear drop in activity.

The results found in the current work are consistent with what was found by Petersen (2007) for trypsin reacted with 5 kDa SC-mPEG, in so far as conjugates produced from immobilised trypsin had higher activity towards azocasein and BAPNA than when free trypsin was PEGylated. Furthermore, the current work shows that the specific activity of PEGylated immobilised trypsin towards both substrates is increased as a function of increasing reaction time from 0-60 min and thus increasing numbers of PEG groups attached.

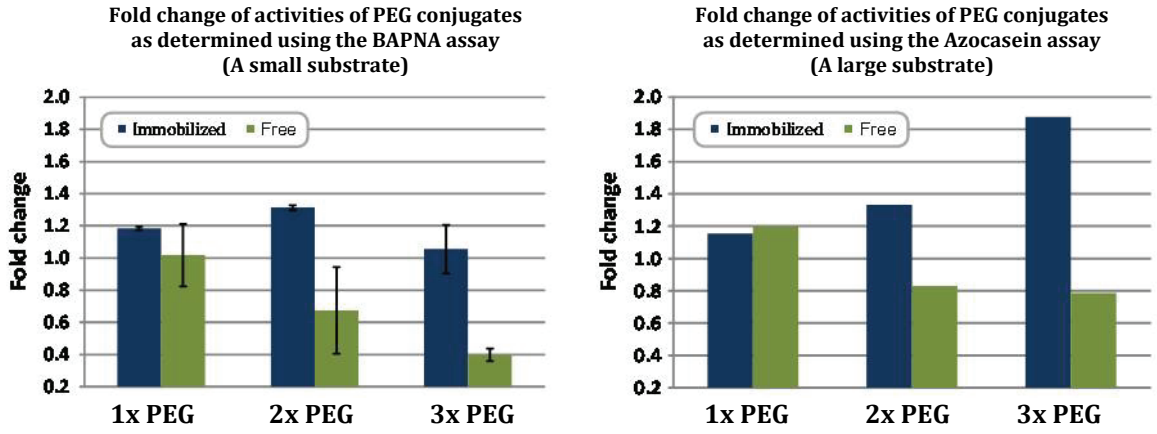

Figure 3.18: Fold change of activities for PEG-conjugates produced with immobilized or free trypsin, for the data presented in Table 3.5. Left figure shows the fold change of activities of PEG conjugates towards the small substrate BAPNA, while the right side shows the activity as measured using the large substrate azocasein.

Other investigators have found similar results to those seen here, and Caliceti et al. (1993) reported that trypsin conjugates prepared when immobilized by benzamidine on Sepharose resins also displayed higher activity when compared to freely PEGylated samples with or without an inhibitor present. However, unlike the results obtained by Petersen (2007), or in the current work, the activity of the conjugates produced by Caliceti and co-workers were always found to be lower as compared to an unPEGylated control sample.

3.3.3 Assembly of a semi-continuous PEGylation process

A major objective of the current work is to examine whether magnetic supports can be used as the basis for a semi-continuous process for protein PEGylation. The principle and potential advantages of using magnetic particles was demonstrated in batch experiments in the previous sections and appropriate buffers, concentrations of reactants and conditions have been identified. The results indicate that all steps require only short time intervals (approximately 5 minutes) thus opening up for the possibility of conducting each step in a series of connected, sequential 'pipe reactors', such as those based on SHM reactors. A schematic of the sequence of operations is shown in Figure 3.19. However, it is to be expected that performance of each of the steps in a pipe reactor may not be as efficient as in a well mixed test tube, especially with viscous solutions and at the small scales to be used. Therefore each of the 3 sequential steps was examined before connecting them together into one complete process.

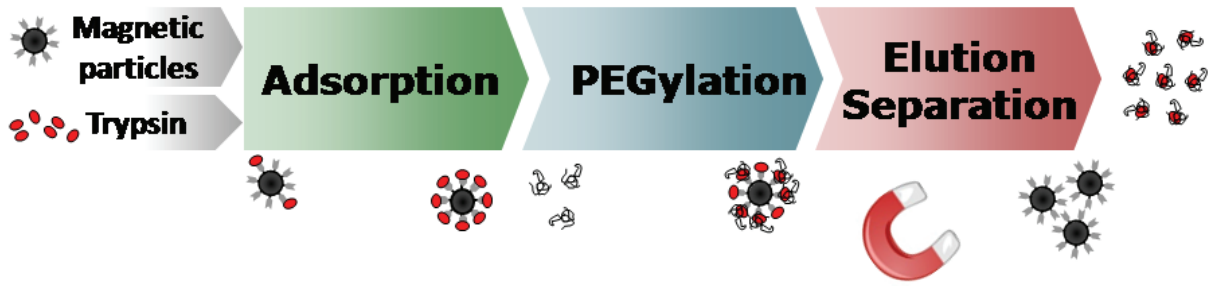

Figure 3.19: The sequence of steps in the proposed semi-continuous PEGylation process. During the first step, designated *adsorption*, the target protein (trypsin) is adsorbed on to benzamidine derivatized magnetic support. Under next step, denoted *PEGylation*, the process of covalent attachment of PEG is conducted. Finally during the last step known as *elution/separation,* the PEG-conjugates are washed, eluted and separated from the magnetic support which may be recirculated during a new process run.

Evaluation of continuous adsorption of trypsin to magnetic supports using staggered Herringbone Mixers

Adsorption of trypsin to benzamidine derivatized magnetic supports is the first step in the semi-continuous PEGylation process. Rapid adsorption requires good mixing of the supports and the trypsin. After a number of preliminary experiments with different prototype pipe reactors, such as those used by Ferré (2005), staggered herringbone mixers (SHM) were selected for further investigation. The experimental setup consisted of a dual syringe pump and a single SHM type unit (see Figure 3.3). Based on the investigation of adsorption kinetics above (Figure 3.8) it was decided to focus on the performance of SHM using residence times of 2.5 and 5 minutes. The SHM-2A and 3A types (Table 3.1), with internal measured volumes of 1 ml and 0.5 ml respectively, were tested individually using a similar approach. The SHM unit was connected to the flow of the magnetic support (4 mg/ml) and the trypsin solution (0 - 4 mg/ml), each of which were fed at 115 µl/min, thus leading to a combined flow of 230 µl/min and 2-fold dilution. From the point of merger between the two flows and to the collection point, the residence times were thus 2.6 minutes for the SHM-type 3A unit and 5.1 minutes for SHM-type 2A. To ensure that the system was stable and all flows were well developed, at least one system volume was allowed to pass before sampling from the outlet. Collection of samples from the system outlet was achieved by directing the particle suspension for 5 minutes down the side of a 15 ml tube attached to a permanent bar magnet (0.5 Tesla). The magnetic particles thus remained stuck to the side of the tube and adsorbent free supernatant collected in the bottom of the tube. The results for the studies are shown in Figure 3.20.

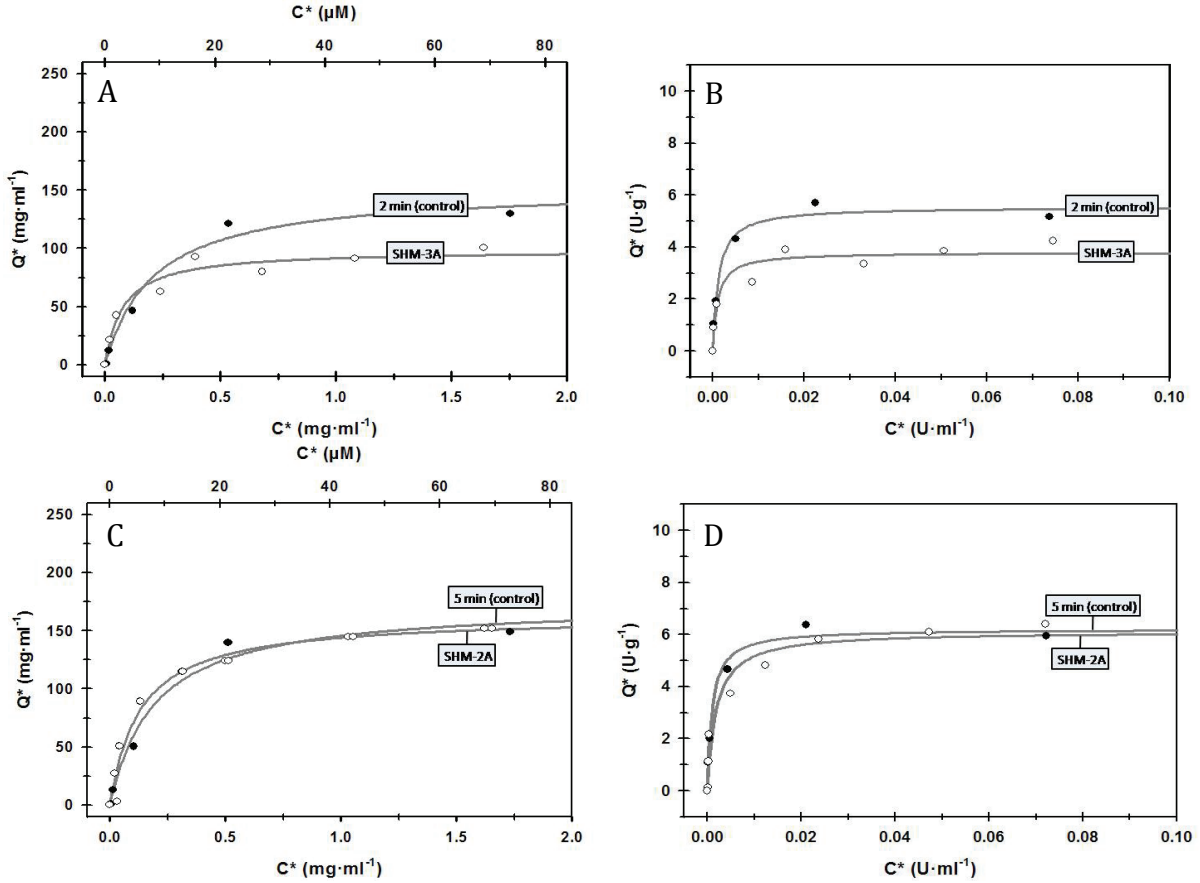

Figure 3.20: Comparison of the performance of the SHM-type units during the adsorption step. All binding studies were conducted in PEG4 buffer. (A+B) Performance of the SHM-3A (0.5 ml) as described by bound protein (A) and by bound proteolytic activity (B). (○) Represents data points collected using the SHM-3A reactor, while (●) are data from a control experiment using microtubes. (C+D) shows the performance of the SHM-2A (1 ml) as described by bound protein (C) and by bound proteolytic activity (D). (○) Represents data points collected using the SHM-2A reactor, while (●) are data from a control experiment using microtubes.

Table 3.6: Langmuir model parameters for isotherms for the SHM-type reactors presented in figure 3.20. Q_{max} represents the maximum binding capacity while k_d indicates the dissociation value (concentration at which 50% capacity is achieved). Q_{Max}/k_d reflects the initial slope of the isotherm and is referred to as tightness of the binding.

	Q_{max}	k_d		Q_{max}/k_d	Q_{max}	k_d	Q_{max}/k_d
	mg/g (Std_{Err})	mg/ml (Std_{Err})	µM (Std_{Err})	l/g	U_{BAPNA}/g (Std_{Err})	U_{BAPNA}/l (Std_{Err})	l/g
SHM-3A	173.2 (12.5)	0.189 (0.052)	7.94 (2.2)	0.92	3.8020 (0.245)	0.0010 (0.0005)	3.8
Reference 2.5 min	98.7 (5.7)	0.0785 (0.023)	3.30 (0.97)	1.3	5.5498 (0.229)	0.0012 (0.0003)	4.6
SHM-2A	162.9 (11.8)	0.1305 (0.036)	5.48 (1.5)	1.3	6.1215 (0.402)	0.0018 (0.0008)	3.4
Reference 5 min	152.7 (11.6)	0.2131 (0.059)	8.95 (2.47)	0.7	6.2141(0.297)	0.0010 (0.0003)	6.2
	Total protein measurements				Trypsin activity (BAPNA)		

Figure 3.20 shows that excellent isotherms could be obtained with both mixer types, which could be fitted with the Langmuir model. However despite this, it was evident that the SHM-2A unit with a 5 min residence time gave the best binding performance. For the SHM-2A, performance was very similar to that seen in well mixed micro tubes incubated for 5 min (see Figure 3.8): Values for Q_{max} were 163 mg/g (6.12 U/g) or 174 mg/g (6.21 U/g), while k_d was 0.1305 mg/ml (1.1 U/l) and 0.1947 mg/ml (1.0 U/l) for the SHM-2A and the 5 minute well mixed tubes, respectively. Values for Q_{max}/k_d were also comparable. In contrast, the performance of the SHM-3A with a 2.5 minute residence time was much worse than that for well mixed tubes with the same incubation time: Q_{max}) was found to be ca. 33% lower (99 mg/g or 3.8 U/gcompared to 153 mg/g or 5.6 U/g) and k_d values were 2.5 fold higher for the SHM-2A. Thus the tightness of binding (i.e. Q_{max}/k_d) was decreased by 2-fold from 0.72 to 1.3 $l \cdot g^{-1}$ and from 2.1 to 4.6 $l \cdot g^{-1}$, for the mixer compared to well mixed tubes, based on total protein and activity measurements, respectively. Based on these findings it was concluded that the SHM-type 2A unit and a fixed 5 minute period of adsorption were best suited for the process step.

Investigation of the PEGylation step using Staggered Herringbone Mixers

To evaluate whether the PEGylation step could be performed using a SHM type mixer unit, a setup was prepared as described in section 3.2.5 and presented in Figure 3.4c. Briefly, the same dual action syringe pump used in the adsorption study and a Staggered Herringbone Mixer type 2B unit (SHM-2B) with an internal volume of 2 ml were employed. Based on the knowledge gained during the microtube based study of the PEGylation reaction, a reaction time of 5 minutes was deemed appropriate and the flow rates of the solutions adjusted accordingly. The process was performed by pumping 2 mg/ml trypsin preloaded magnetic support and various SC-mPEG concentrations both at 226 µl/min, resulting in a combined flow of 452 µl/min. This gave a process time corresponding to 5.1 minutes when a 40 cm x 1 mm internal diameter exit tube was used after the mixer. Termination of the reaction and collection of the immobilized trypsin conjugates was achieved by directing the emerging flow into a collection tube containing 10 ml PEGylation buffer supplemented with 50 mM lysine. In all experiments

the magnetic supports were preloaded with trypsin to a capacity of ca. Q= 150 mg/g. To ensure the system was stable, the process was allowed to proceed for 6 minutes before samples were collected. Reference microtube based PEGylation experiments were also prepared using similar reaction times, preloaded support and SC-mPEG concentrations and both sets of processed magnetic beads were subsequently washed and eluted, and the samples subsequently analyzed using both reduced SDS-PAGE and MALDI-TOF (Figure 3.21 and 3.22).

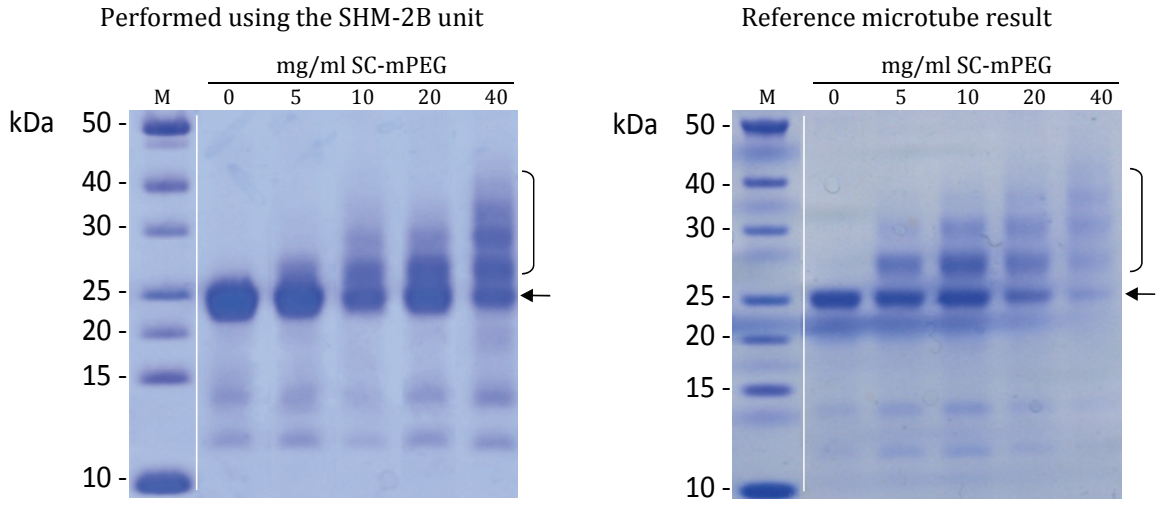

Figure 3.21: Reduced SDS-PAGE gels showing elution fractions following PEGylation of immobilized trypsin using various concentrations of 2 kDa SC-mPEG. Left gel shows the results obtained from the SHM-based process, right shows the results of reference microtube based experiment using similar conditions. The arrow indicates the native trypsin band while the bracket indicates the position of the PEG conjugates formed. The employed SC-mPEG concentrations are noted at the top. 'M' = molecular weight markers.

As can be seen in Figure 3.21, the results of the SDS-PAGE analysis show the same PEGylation profiles for all the reactions conducted in the SHM-2B system and those conducted in well mixed microtubes. In both cases higher PEG concentrations led to conjugates with more PEG groups attached, as was also seen in earlier trials (Figure 3.9). For the reactions carried out using 5 mg/ml SC-mPEG two bands were visible on the SDS-PAGE gel corresponding to native trypsin and the formation of conjugates carrying a single PEG group (Figure 3.21).

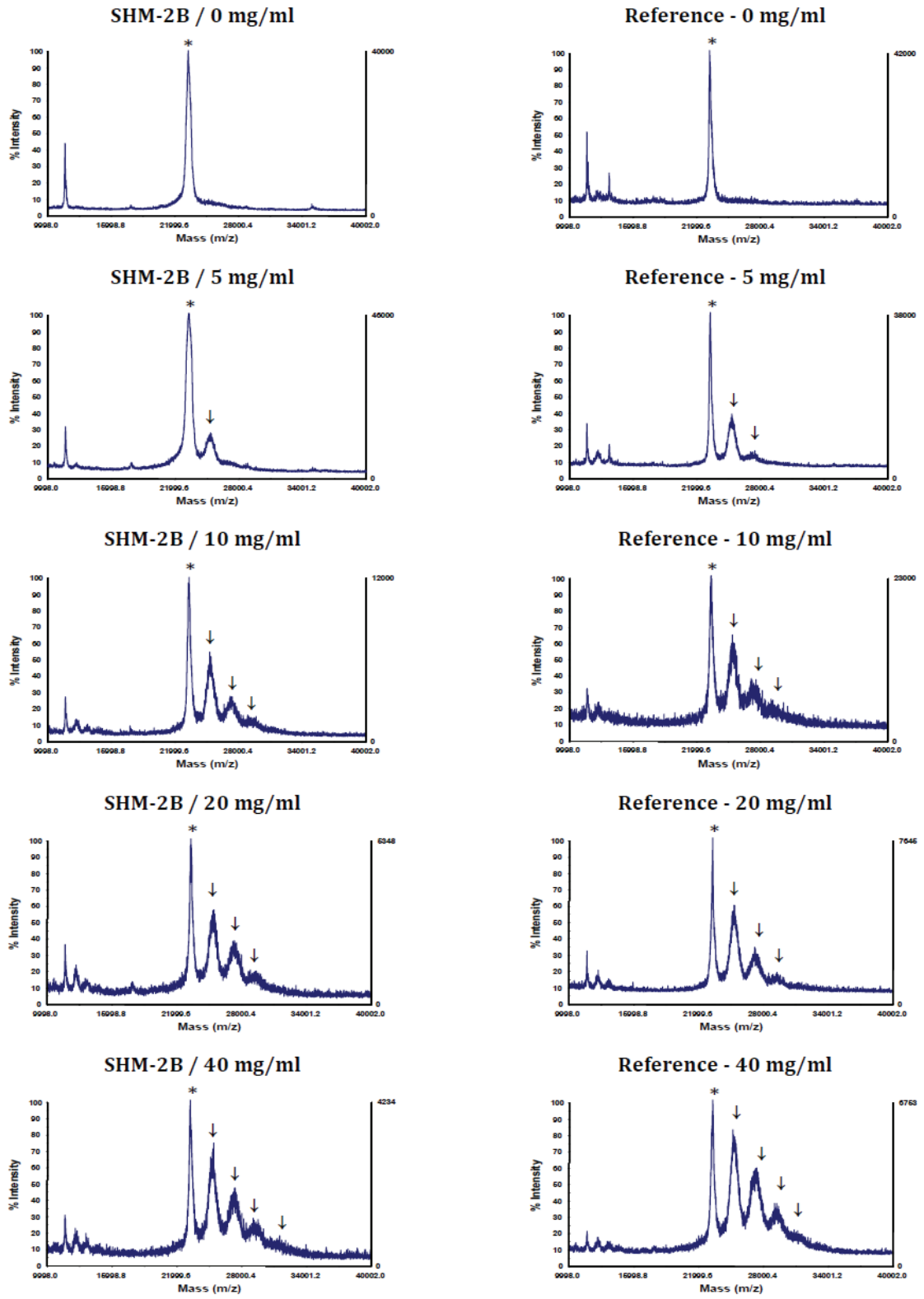

Figure 3.22: MALDI-TOF spectra comparing PEGylated samples originating from the SHM-2B based continuous process or from reference microtube based samples. (*) indicates the native trypsin peak while arrows shows the position of peaks corresponding to PEG conjugates.

Samples originating from reactions conducted using 10 mg/ml and 20 mg/ml SC-mPEG all show a remarkable resemblance and a total of three bands could be observed on the SDS-PAGE gels, reflecting the presence of two species of positional PEG isomers carrying one or two PEG groups, as well as the band corresponding to native trypsin. The samples generated using 40 mg/ml SC-mPEG revealed the presence of four PEG-conjugate derived bands representing species with 1-4 attached PEG groups. Further analysis using MALDI-TOF spectra (figure 3.22) confirmed the interpretation from the SDS-PAGE results. On the basis of these results it was concluded that the Staggered Herringbone mixer type 2B with a 5 minute residence time was suitable for the PEGylation of immobilised trypsin in a semi-continuous process.

Evaluation of the semi-continuous wash and elution step

The final part of the semi-continuous PEGylation process involved a wash and elution step during which excess reagents were removed, the PEG conjugates eluted and the magnetic support separated from the product. However, unlike the previous steps in the process, which could be performed in a continuous mode, this part of the process could only be performed batch-wise due to the technical limitations of the HGMS magnetic separator setup described under section 3.2.5 and shown in Figure 3.26D. To investigate the performance of this step, 40 mg magnetic supports carrying PEGylated conjugates of trypsin prepared using 150 mg/ml SC-mPEG in microtubes, were loaded directly into the magnetic filter while the field was on. Three rounds of washing were subsequently performed using 20 ml of PEG4 buffer in each wash, followed by three serial rounds of elution each carried out using 12 ml of elution buffer. The eluted samples were analyzed using SDS-PAGE and the results showed that only the first sample contained PEG-conjugates, indicating that a single elution step was sufficient to recover the product.

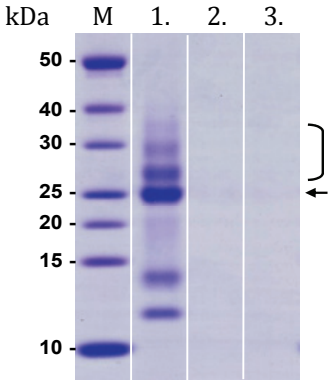

Figure 3.23: Reducing SDS-PAGE analysis of three serial elution steps performed using the semi-continuous HGMF system. The number at the top of each lane reflects the number of the elution. The arrow indicates the native trypsin band while the bracket indicates the position of the PEG conjugates formed.

During microtube based PEGylation experiments, as many as three consecutive elution steps were needed to recover all the PEGylated products and two steps were routinely used by Petersen (2007). However, as is seen in the Figure 3.23 a single step elution was sufficient in the current work. The observed increased efficiency of the elution step, when performed using the semi-continuous setup, was unexpected and may be connected to the slow release of the magnetic support from the magnetic filter. Initial tests to evaluate the performance of the magnetic filter employed in the setup (see section 3.2.5) revealed that the support was retained with high efficiency and that the subsequent release from the filter only proceeded slowly as compared to capturing process.

Evaluation of the full HGMF-based semi-continuous PEGylation process

The individual components examined in the previous sections were assembled into one complete sequential process (see figure 3.4 and figure 3.26) consisting of: (i) continuous adsorption of trypsin to magnetic particles in the SHM-2A mixer, which was connected to, (ii) continuous PEGylation in a SHM-2A mixer that in turn was connected to, (iii) continuous reaction termination using 50 mM lysine in a SHM-2A mixer and the immobilised PEGylated trypsin was subsequently captured directly in a HGMF separator and processed (see section 3.2.5 for more details). All solutions were prepared in PEG4 buffer. The concentration of magnetic support and the trypsin solutions used during the characterization of the process were 4 mg/ml and 1.2 mg/ml, respectively. The concentration of activated PEG solution used was 0, 10, 20 or 40 mg/ml and was in all cases prepared as 2x solution compared to the concentration needed during the PEGylation reaction.

To ensure stabilisation of the entire system had occurred, the process was allowed to proceed for 15 minutes before any samples were gathered. To test for stabilisation of the system, a small sample of the support was also secured prior to initiation of collection by the magnetic filter in the HGMS system. In addition to this, a sample was also obtained after the HGMS based collection was terminated. In both cases the samples were washed and eluted in microtubes. The results are shown in figures 3.24 and 3.25.

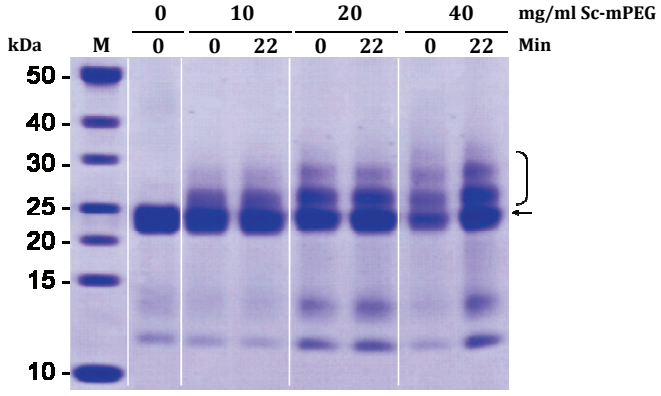

Figure 3.24: Reduced SDS-PAGE analysis showing the PEG profiles of conjugates eluted from magnetic support secured immediately after the process was started (0 min) and after the complete process had been running for 22 minutes (22 min). The arrow indicates the native trypsin band while the bracket indicates the position of the PEG conjugates formed. The SC-mPEG concentrations employed are noted at the top.

SDS-PAGE analysis of the samples produced from the complete process visualized in figure 3.24 showed excellent performance of the system over the 22 minute period examined. The profile of the PEG conjugates produced could be controlled by varying the concentration of activated PEG used, delivering conjugates with 1, 2, or 3 groups attached (Figure 3.24). The system also showed excellent stability, the PEG conjugate profiles after 22 minutes of processing were the same as those produced immediately after the process was started. Furthermore, the profile of PEG conjugates is as expected from the preliminary trials (Figure 3.21). The complete semi-continuous PEGylation process was also performed for a 40 minute period to generate larger amounts of PEG conjugates. SC-mPEG concentrations up to 40 mg/ml were used and the PEG-conjugates produced (Figure 3.25) were consistent with those seen in the process run for 22 minutes (Figure 3.24).

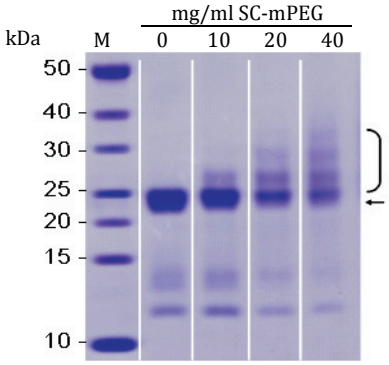

Figure 3.25: Trypsin-PEG conjugates produced by the full HGMF-Based semi-continuous PEGylation process. The arrow indicates the native band while the bracket indicates the position of the PEG conjugates formed. The SC-mPEG concentration employed is noted at the top.

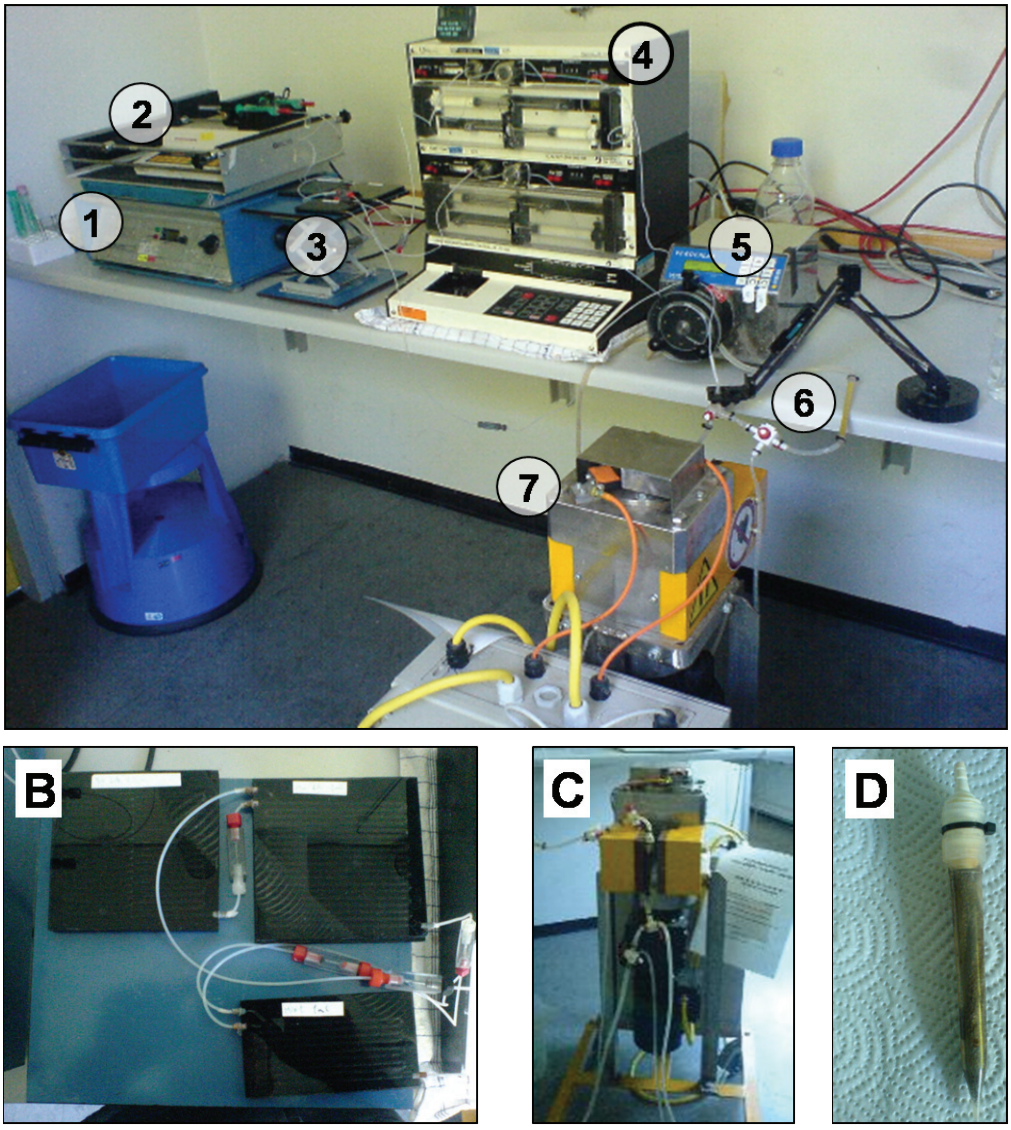

Figure 3.26: The semi-continuous PEGylation system. (A) Shaking table(1), Dual action syringe pump (2), SHM-units (3), Reciprocal pumps (4), Peristaltic pump (5), Inline static mixer (6) and HGMS (7) unit. 1-4 is part of the continuous parts of the process, while 5-7 is included in the discontinuously operated separation and elution step. (B) Close up of the interconnected SHM-units and (C) The HGMS unit and (D) the cone-shaped magnetic filter, which was placed in the HGMS unit.

3.4 Conclusion

A HGMF-based semi-continuous process for controlled modification of proteins has been demonstrated for the first time. Trypsin, which was reversibly immobilized on the surface of non-porous benzamidine derivatized magnetic supports could be PEGylated in a semicontinuous fashion and delivered defined populations of PEG-conjugates. Each of the steps in the complete process could be optimised separately and assembled into a final process, which exhibited the performance expected. A key to the success of the complete process was ensuring adequate mixing in each stage, which in this case, was accomplished using staggered herring bone mixers.

It was established that protein adsorption to the magnetic supports was a fast process and that control of the PEGylation process could be achieved by tight regulation of reaction time as well as concentration of the activated PEG. Termination of the reaction was best achieved by addition of lysine, which provided a source of free amino groups for reaction with excess activated PEG. Comparison of the activity of conjugates prepared using immobilized trypsin and enzyme in free solution supported the hypothesis proposed by Petersen (2007) that immobilization via the active site preserves enzyme activity and serves to promote formation of conjugates displaying positive effects of PEGylation.

The PEGylation process developed here was in itself fully continuous but was operated in a semi-continuous way due to the batch wise nature of the HGMF part. Development of continuous HGMF processing would allow fully continuous PEGylation. Extension of the process developed here for other types of biomolecule modification or production-line assembly of biological complexes should be considered.

Acknowledgements

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Chapter 4

Assembly of a nano-engine using magnetic particles

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4. Assembly of a nano-engine using magnetic particles

4.1 Introduction

In the previous chapter it was successfully demonstrated that magnetic supports can be used as the basis for a semi-continuous process for controlling protein PEGylation. The process revolved around sequential continuous steps in which the protein was first temporarily anchored to the magnetic support, then modified, and the reaction quenched before discontinuous HGMF processing for recovering the modified protein. In previous work, Ferré (Ferré, 2005A/B) showed that a similar semi-continuous HGMF based process could be used for protein refolding. These works indicate that the magnetic support may be a generic, convenient means of manipulating proteins in a variety of environments and for a much wider variety of applications than most other works have focused on, *i.e*. not only for downstream processing. Extending the concept developed in the previous chapter further, it could be envisaged that the magnetic particle may provide a support for the continuous and scalable assembly of protein complexes in much the same way that is used in the assembly line manufacture of e.g. cars and other complex mechanical devices composed of numerous components, which need to be put together in a specific order. For example a protein complex made up of three subunits could, in principle, potentially be assembled using the process developed in the proceeding chapter (see figure 3.4a). The anchoring of a base protein to the support would be step 1 (i.e. replacing trypsin adsorption), addition of the first subunit(s) for assembly would be step 2 (i.e. replacing the PEGylation reaction) and addition of the third subunit(s) would be the final step (i.e. replacing the termination reaction in figure 4.3a), before using HGMF to recover the purified protein complex in solution. To explore the potential of magnetic supports for assembly line manufacture of protein complexes, the nano-bio motor based on the F_oF_1 ATP synthase was chosen. Thus the overall aim of the study presented in this chapter was to examine whether magnetic particles could be used as smart handles for the assembly of the F_oF_1-ATP synthase.

The F_oF_1 ATP synthase, also known as the F-type ATPase (EC 3.6.3.14) is a ubiquitous, abundant and multimeric protein complex required for the coupling of the electrochemical gradient formed by the respiratory chain (oxidative phosphorylation) or photosynthetic electron transport and the synthesis of ATP (Senior 1988). The complex is found in the mitochondrial inner membrane, the inner membranes of bacteria and the thylakoid membrane of chloroplasts and belongs to the enzyme class of acid anhydride hydrolases (EC 3.6). The F-type ATPase is structurally divided into two very distinct regions (Figure 4.1), namely a membrane bound part denoted F_0 and a membrane peripheral part known as F_1, connected by a slender stalk (Walker and Collinson, 1994).

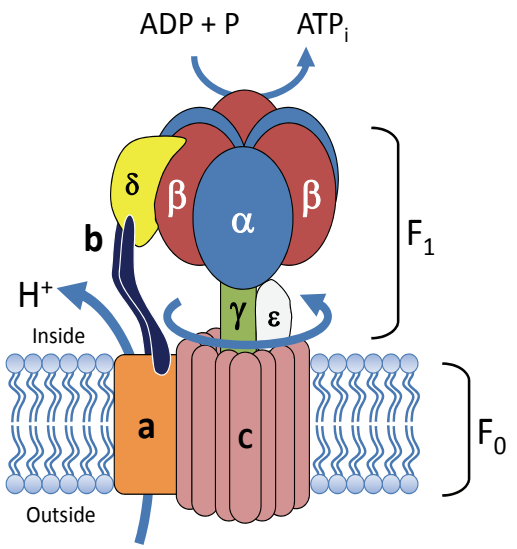

Figure 4.1: Structural model of the *Escherichia coli* F_0F_1-ATPsynthase. The F_0F_1-ATP synthase consists of eight different subunits arranged in two distinct parts denoted F_0 (a, b, c) and F_1 (α, β, γ, δ, ε) containing at least 24 proteins. The figure is based on Pedersen et al. (2002).

The total number of distinct subunits found in connection with the F_0F_1-ATP synthase complex differs from organism to organism, and may exceed more than twenty in eukaryotes. For the bacterial F-type complex the number is limited to only eight. However, despite differences in both the total number of subunits as well as the unique components, structural investigations have revealed a remarkable similarity and this suggests that the mode of action is highly conserved through evolution (Wilkens 2000, Pedersen et al., 2000). Computer aided modeling studies combined with x-ray crystallography structures have revealed that the F_1-ATP synthase complex is highly monodispersed. The α/β-subunits are placed on a stalk like structure composed of the γ subunit, which in turn serves as connecting link to the F_0 part of the complex (Figure 4.1). The δ-subunit interacts with the α/β structure, while the ε-subunit is located at the base of the Stalk and appears also to be connected via the γ subunit (Wilkens et al. 1995, 1997, 2000). The coupling between the synthesis of ATP and the charge gradient has been well studied and the energy required for the generation of ATP is harvested by a downhill flow of protons through the F_0 domain, which results in a physical rotation of

the complex. The rotating action and the torque created by the harvested energy causes an alteration of the β-subunits enabling the formation of ATP from inorganic phosphate and ADP. Under conditions where the F_1 part is dissociated from the F_0 domain, the reverse reaction is favoured and the energy released by the hydrolysis of ATP causes a transfer of mechanical energy in the opposite direction (Kinosita et al. 2000; Yoshida et al. 2001). A direct observation of the rotation of the F_1-ATP synthase from the thermophilic bacteria *Bacillus PS3* was published in 1997 by Noji and co-workers (Noji et al. 1997) in an intriguing study in which the complex was immobilized on a glass surface. Through the attachment of an actin filament carrying a fluorescent probe it was observed that a full 360° rotation occurred in three discrete 120° steps, and that each step required hydrolysis of a single ATP molecule. It was extrapolated that the rotational torque produced by the rotary movement reached an average of more than 40 pN·nm. This observation was later confirmed by Kinosita and co-workers (2000) who reported a similar value and in addition to this calculated the energy efficiency to be approximately 90%, making the F1-ATPase not only the smallest known "engine", but also the most energy efficient. The described mode of action by the F_1-ATPase has led researchers to compare the complex to the internal rotary combustion engine invented by Felix Wankel in 1957, known as the Wankel engine (Figure 4.2).

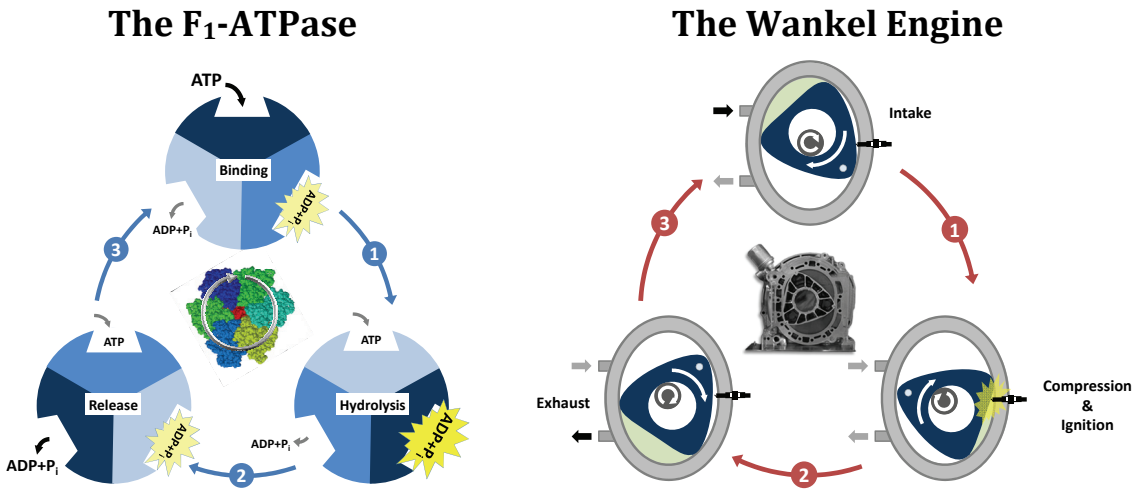

Figure 4.2: Comparing the action of the ATPase and the Wankel engine. The rotational torque created by the wankel engine is produced by internal combustion, while the rotating action of F_1-ATPase complex is mediated by hydrolysis of ATP. However, both engines have three "reaction chambers" and both work using three cyclic steps, which on close inspection show a high degree of similarity. The figure is based on Yoshida et al. (2001).

A review of the literature reveals that *in vitro* reconstruction of the F_1-part has been successfully achieved in several reports dating as far back as 1977, where Futai and co-workers (1977) successfully reassembled a complex consisting the α, β and γ subunit and demonstrated that this minimal subset of components were enough to achieve ATP hydrolyzing activity. However, reconstructed F_1-ATPase consisting of α, β , γ and the δ or ε subunits was also reported by Shin et al. (1996). In that study, reassembly was achieved by initial immobilization of the δ- and ε-subunits via an amino-terminal GST-tag using standard glutathione-sepharose chromatography material and it was demonstrated that the δ subunit promotes activity, while the presence of the ε appeared to have the opposite effect.

Inspired by the above reports and other studies, three assembly strategies were devised around the use of different means for anchoring the first base subunit to a magnetic support, to which the other subunits could then self assemble. The strategies centered around the use of different affinity tags (histidine affinity tag, maltose binding protein, and steptavidin binding domain) for reversible anchoring to magnetic supports derivatised with different ligands (iminodiacetic acid, amylose or biotin), as well as the tagging of different subunits to test which may be best suited for acting as the first base subunit (i.e. □, □, □, □ or □). To obtain pure forms of the subunits associated with the F_1-ATPase complex (alpha, beta, gamma, delta and epsilon) the initial work involved preparing plasmids harbouring recombinant forms of the subunits. The presence of the poly-histidine tags permitted purification by metal chelate affinity chromatography after bacterial overexpression. Subsequently the basic principle used during the assembly processes involved initial adsorption of a single central subunit serving as the scaffold for the assembly of the remaining complex. A more detailed description of the individual strategies associated with the various support types used follows in the results section, while a discussion of the strategy connected with the choice of tags and their actual position within the primary sequence is provided in appendix C.

4.2 Materials and methods

4.2.1 Chemicals and materials

All Chemicals including the GenElute Plasmid Miniprep Kits used in the study were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO 63103, USA) unless otherwise mentioned. The *E. coli* expression strain BL21(DE3) was purchased from Stratagene (11011 N. Torrey Pines Road La Jolla, CA 92037, USA) as ready made competent cells. The *E. coli* strain DH5α used for DNA manipulation was obtained from an in-house stock and made competent using the method described by Inoue et al. (1990). Enzymes used in connection with DNA manipulations and the expression vector pTwin1 were obtained from NEB (New England Biolabs, Ipswich, MA 01938-2723, USA), while Phusion High-Fidelity DNA Polymerase (F-530) was obtained from Finnzymes (Finnzymes Oy, 02150 Espoo, Finland). Primers was purchased online from Sigma-Aldrich, while sequencing was performed using the mail/web service provided by MWG-Biotech (Eurofins MWG Operon, 85560 Ebersberg, Germany / www.mwgdna.com). Chelating Sepharose Fast Flow chromatography media and the GFX PCR DNA/Gel Band Purification Kit were acquired from GE Healthcare (GE Healthcare Bio-Sciences AB, SE-751 84 Uppsala, Sweden). Amylose embedded magnetic beads and DNA manipulating enzymes were acquired from NEB (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA), while prototype biotin derivatized magnetic particles were received as a gift from Priv. Doz. Dr.-Ing. Matthias Franzreb (Institute for Technical Chemistry, Forschungszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany). Neodymium block magnets were obtained from Danfysik A/S (Jyllinge, Denmark) and Hindsbo magneter Aps (Roskilde, Denmark), and magnetic racks were made in-house. Precast SDS-PAGE gels, AcTEV protease and buffers were obtained from Invitrogen (Carlsbad, California, USA). The BCA assay and Coomassie Plus Bradford Assay reagents were purchased from Pierce Biotechnology (Thermo Fisher Scientific, Illinois, USA). All DNA manipulation was performed according to instructions given by the respective manufactures of the kits or reagents used, and in accordance with the methods described by Sambrook et al. (2001).

4.2.2 Manufacturing of IDA-derivatized magnetic support

The aminosilane terminated magnetic base matrix was prepared as described in chapter 2. Briefly, they were prepared from iron(II)/iron(III) chlorides and 3-aminopropyltriethoxysilane, according to the procedures described by Hubbuch and Thomas (2002). Polyglutaraldehyde coating was then conducted as described by O'Brien et al. (1996), while subsequent attachment of the allyl glycidyl ether link, bromination and coupling of iminodiacetic acid were carried out as described by Heebøll-Nielsen (2002). Particles were stored in storage buffer (20 mM sodium phosphate, 1 M NaCl, pH 6.8) at 4°C until required. Particle concentrations were determined using a dry weight method which involved desalting the particles by washing followed by incubation overnight at 95°C in pre-weighed glass tubes.

4.2.3 Binding of denatured H□□subunit to copper charged IDA-magnetic supports.

A series of solutions containing various known concentrations of denatured Hγ (8 M urea) ranging from 0 to 0.6 mg/ml were prepared, and 0.5 ml was added to 2 mg copper(II) pre-charged IDA-magnetic particles. The resulting suspension was subsequently incubated with vigorous shaking for 30 minutes at room temperature, after which the magnetic support was separated by centrifugation and a sample of the supernatant collected using a pipette. The concentration of protein in the supernatant was subsequently determined using the BCA assay.

4.2.4 Buffers and medium

Assembly buffer: 50 mM succinate-Tris, pH 6, 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 10% (w/v) glycerol. *Denaturation buffer:* 100 mM Na_2HPO_4, 10 mM Tris-HCl, 8 M urea, pH 8. *PBS buffer:* 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.24 g/l KH_2PO_4, pH 7.4. *LB medium:* 1% w/v Bacto yeast extract, 0.5% w/v tryptone, 1% w/v NaCl. The media was autoclaved prior to use. Optional addition of 100 mg/l ampicillin was done after autoclavation. *SOC medium (transformation):* 2% w/v bacto-tryptone, 0.5% w/v Bacto Difco yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20 mM glucose. pH was adjusted to 7 using 1 M NaOH. Glucose was autoclaved separately. *MBP*

Binding Buffer: 200 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.4. *MBP Elution Buffer:* 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.4, 10 mM maltose.

4.2.5 Assembly of the ATPase on magnetic metal chelate adsorbents

To achieve assembly of the ATPase on magnetic metal chelate adsorbents, 2 mg IDA-mPGA support was charged with copper(II) as described above and subsequently incubated with 1 ml denaturing buffer (100 mM phosphate, 10 mM This, 8 M urea) containing 0.6 mg/ml Hγ. After a 30 minute incubation period under vigorous shaking, the magnetic support was washed three times in denaturing buffer. At this point 200 µl of a denaturing buffer containing 2 mg/ml Hα and 2 mg/ml Hβ was added to the immobilized Hγ and the resulting suspension transferred to a tube containing 12 ml of a reconstruction buffer consisting of 50 mM succinate-tris (rapid dilution), pH 6 supplemented with 5 mM ATP, 5 mM $MgSO_4$, 10% w/v glycerol and 0.5 mM EDTA. The resulting suspension was afterwards incubated overnight at room temperature under rotation and subsequently washed twice in reconstitution buffer to remove excess protein, followed by an elution step performed using a 200 µl 50 mM Tris-buffer, pH 7.6 supplemented with 250 mM imidazole.

4.2.6 Analytical methods

ATPase activity: The ATP hydrolyzing activity was determined by mixing 100 µl of the samples with 900 µl of 100 mM Tris-HCl buffer, pH 7.5, supplemented with 5 mM ATP and 5 mM $MgCl_2$. At predetermined time intervals a 200 µl sample was taken and the activity was stopped by addition of 20 µl 50% (w/v) TCA. The content of inorganic phosphate was subsequently determined using the P_i colorLock Gold system (Innova Biosciences Ltd., Cambridge CB22 3AT, UK) as described by the manufacture.

HPLC: Ion-pair HPLC chromatography for ATP and ADP determination was performed using an Agilent HPLC 1100 system (Technologies, Santa Clara CA 95051, USA) equipped with a 10 cm Luna C-18 column (Phenomenex, Torrance, CA 90501-1430, USA) and DAD detector. The flow rate was fixed at 0.3 ml/min and the temperature set at 40°C. Phase I was composed of 100 mM sodium phosphate (pH 8)

supplemented with 10 mM tetrabutylammoniumchloride and phase II consisted 10% H_2O and 90% methanol. At the initiation of the procedure the flow was mixed 90:10 (Phase I/Phase II) and stepwise reversed over a period of 10 minutes. Prior to the analysis, samples containing pure ADP or ATP were used to identify their corresponding peaks and it was established that the retention time was approximately 4.5 minutes and 6.5 minutes, respectively. Based on this knowledge the Chemstation software package (LC-3D, rev. B.01.01/164, Agilent Technologies) was used to identify and integrate the area under peaks using the automated option.

Phosphate assay: To determine the content of inorganic phosphate in samples and to establish possible alterations caused by F_1-ATPase mediated hydrolysis of ATP and subsequent release of phosphate, the P_i ColorLock Gold kit (Innova Biosciences Ltd., Babraham, Cambridge CB22 3AT, UK) was used according to the manufacturers instructions, except that it was conducted in microtiter plates . Adsorption of the resulting samples was determined using a 96-well MultiScan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA 02454, USA). In each series of measurements a standard curve was prepared using the phosphate standard solutions supplied.

SDS-PAGE: Preparation of protein samples for reducing SDS-PAGE was conducted by addition of 5% v/v mercaptoethanol and 1/4 volume 4x NuPAGE LDS sample buffer (Invitrogen, Carlsbad, California, USA) followed by 10 minutes of heating at 96°C. Reducing SDS-PAGE was performed using the NuPAGE Novex Bis-Tris precast gel system from Invitrogen combined with MES SDS running buffer according to the manufactures reccomendations. The protein bands were visualised with Coomassie blue staining. The SDS-PAGE gel was incubated with shaking for 15 minutes in staining solution (0.35 g/l CRR R-250, 45% v/v Ethanol, 9% v/v Glacial acetic acid) pre heated to the brink of boiling in a microwave oven. Background color removal was performed by incubating for 60 minutes in a microwave oven pre-heated destaining solution (10% v/v 2-propanol, 10% v/v Glacial acetic acid) or until the protein bands were visible. Gels were then incubated overnight in distillated water with shaking. Gel images were captured using a flatbed scanner (D660U Canon Inc., Tokyo, Japan). Precast SDS-PAGE gels were obtained from Invitrogen (Carlsbad, California, USA).

4.2.7 Preparation of ATPsynthase subunit expressing plasmids

All plasmids prepared were based on the commercially available vector pTwin1 (part of the IMPACT system from NEB), which carried modified Ssp DnaB (2) and Mxe GyrA inteins flanked by chitin binding domains, which in all cases were removed and replaced by the insert holding the reading frames encoding the recombinant constructs. The T7 promoter was retained in all the constructs prepared enabling controlled expression by the T7 RNA polymerase through addition of isopropyl β-D-1-thiogalactopyranoside (IPTG).

Subunits alpha, beta and gamma: Prior to the preparation of the DNA constructs expressing the recombinant amino-terminal tagged ATP synthase subunits, which included the alpha, beta and gamma, a common precursor vector denoted pTwin1-HAT was prepared. The vector was created by insertion of a small DNA fragment encoding the 19 residue HAT tag (KDHLIHNVHKEEHAHAHNK)[5], an AcTEV protease specific sequence (ENLYFQG) and an alanine linker (3x ala) made from the primer set HAT-fw/HAT-rv. The pTwin1 vector was double digested with *NdeI* and *NotI* resulting in a 6.9 kb fragment, which subsequently was purified from an agarose gel using the GFX system. Before the ligation was performed, the mixture of the primers was heated to 95°C and subsequently cooled, promoting the formation of dsDNA with *NdeI-NotI* compatible ends. To create plasmids pTwin1-Hα, pTwin1-Hβ and pTwin1-Hγ , PCR reactions were performed using plasmid pSH1 (kindly provided by S. Helmark, Department of Systems Biology, Technical University of Denmark) as template (pSH1 carried the sequences encoding the *E.coli* α,β and γ subunits). The primer sets HATA-Fw /A-Rv were used to obtain a 1.6 kb PCR product encoding the alpha subunit, while HATB-Fw/B-Rv and HATG-Fw/G-Rv were used to make 1.5 kb and 1.0 kb fragments harbouring the beta and gamma genes, respectively. All PCR products were subsequently cleaned using the GFX kit, digested with *NotI* and *BamHI* and inserted into a 5.9 kb gel purified fragment originating from pTwin1-HAT, likewise digested with the same set of restriction enzymes. The resulting plasmids were initially analyzed using restriction fragment length polymorphism and subsequently verified by DNA

[5] The HAT tag was based on the sequence contained in the pHAT10 vector from Clontech (AAG GAT CAT CTC ATC CAC AAT GTC CAC AAA GAG GAG CAC GCT CAT GCC CAC AAC AAG)

sequencing. In addition to pTwin1-Hγ a construct designated pTwin1-γ expressing the native gamma subunit was also prepared using the primer set G-Fw/G-Rv and likewise using pSH1 as template. The resulting 0.9 kb purified PCR product was digested with NdeI and BamHI, and inserted into a 5.9 kb gel extracted fragment obtained by treatment of pTwin1 using the same mixture of endonucleases.

Subunits delta and epsilon: DNA constructs expressing chimeric products consisting of a functional minimal streptavidin (SA) domain sandwiched between a truncated allele of the *E.coli* maltose binding protein (MBP) and either the ATP synthase subunit delta or epsilon, were also prepared using the technique of splicing by overlap extension by PCR (SOE by PCR). The fused constructs were prepared with the aim of including multiple tag options (MBP, SA, HAT) allowing binding to be performed with a wide selection of available magnetic particles. Besides this, the included MBP tag also played the role of increasing the solubility of its fusion partner, which is especially needed in the case of the streptavidin tag (Sørensen et al., 2003). To prepare the recombinant constructs, several PCR reactions were performed using the following approach. A 1.7 kb PCR product encoding the MBP and SA domains as well as C-terminal 6xHis tag, was generated using the primer set MBPSA-Fw/MBPSA-Rv and plasmid pET15b-MBP-SA as template (kindly provided by K.K. Mortensen, Department of Molecular Biology, Aarhus University, Denmark). In addition to this, two separate PCR reactions were also performed using the primer sets D-Fw/D-Rv and E-Fw/E-Rv combined with *E.coli* cells crudely lyzed with heat, resulting in the formation of a 0.6 and 0.4 kb product holding the genes encoding the delta and epsilon subunits, respectively. All PCR products were subsequently purified using the GFX system and used as templates during SOE by PCR, which included 10 rounds of initial splicing of the two DNA fragments (1.7/0.6 kb or 1.7/0.4 kb) using an annealing temperature of 60°C in the absence of primers, followed by 20 rounds of amplification of the fused DNA using the primers sets MBPSA-fw/D-rv or MBPSA-fw/E-rv. A schematic presentation of the cloning procedure is available in Figure 4.3, while a schematic presentation of the recombinant translated products is available in Figure 4.4. In addition to this, more details on the plasmids prepared as well primer sequences are available in appendix C.

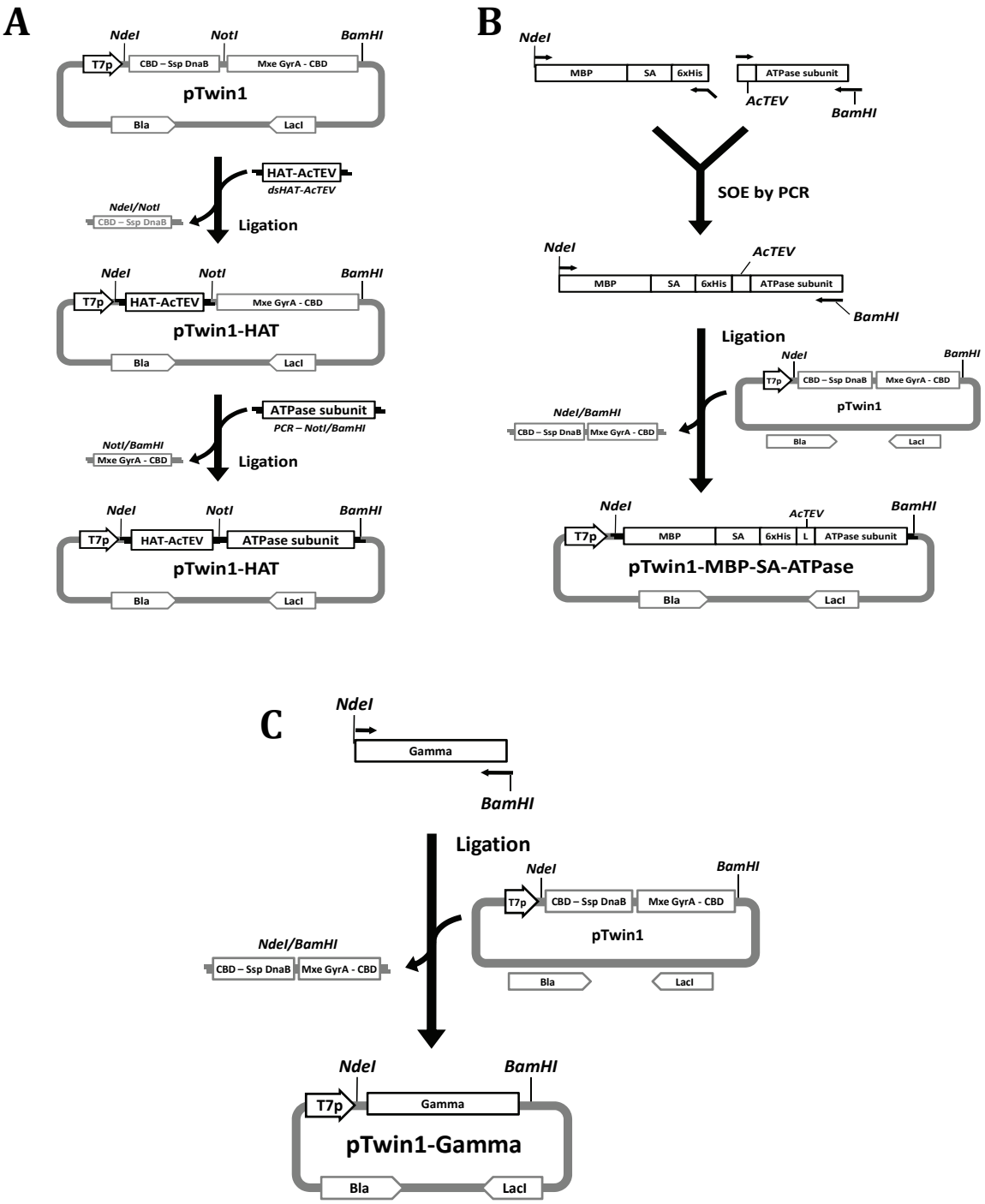

Figure 4.3: Schematic overview of the preparation of plasmids used in this study. (A) The construction of the HAT-tagged ATP synthase subunits (#: Hα, Hβ and Hγ). **(B)** Preparation of the chimeric δ and ε constructs. **(C)** Illustration of the strategy used for making the plasmid enabling over expression of the native γ-subunit. Abbreviations: Bla = ß-Lactamase, T7p = T7 promoter, MBP = Maltose Binding Protein, SA = Streptavidin, HAT = Histidine Affinity Tag, AcTEV = Modified Tobacco Etch virus protease

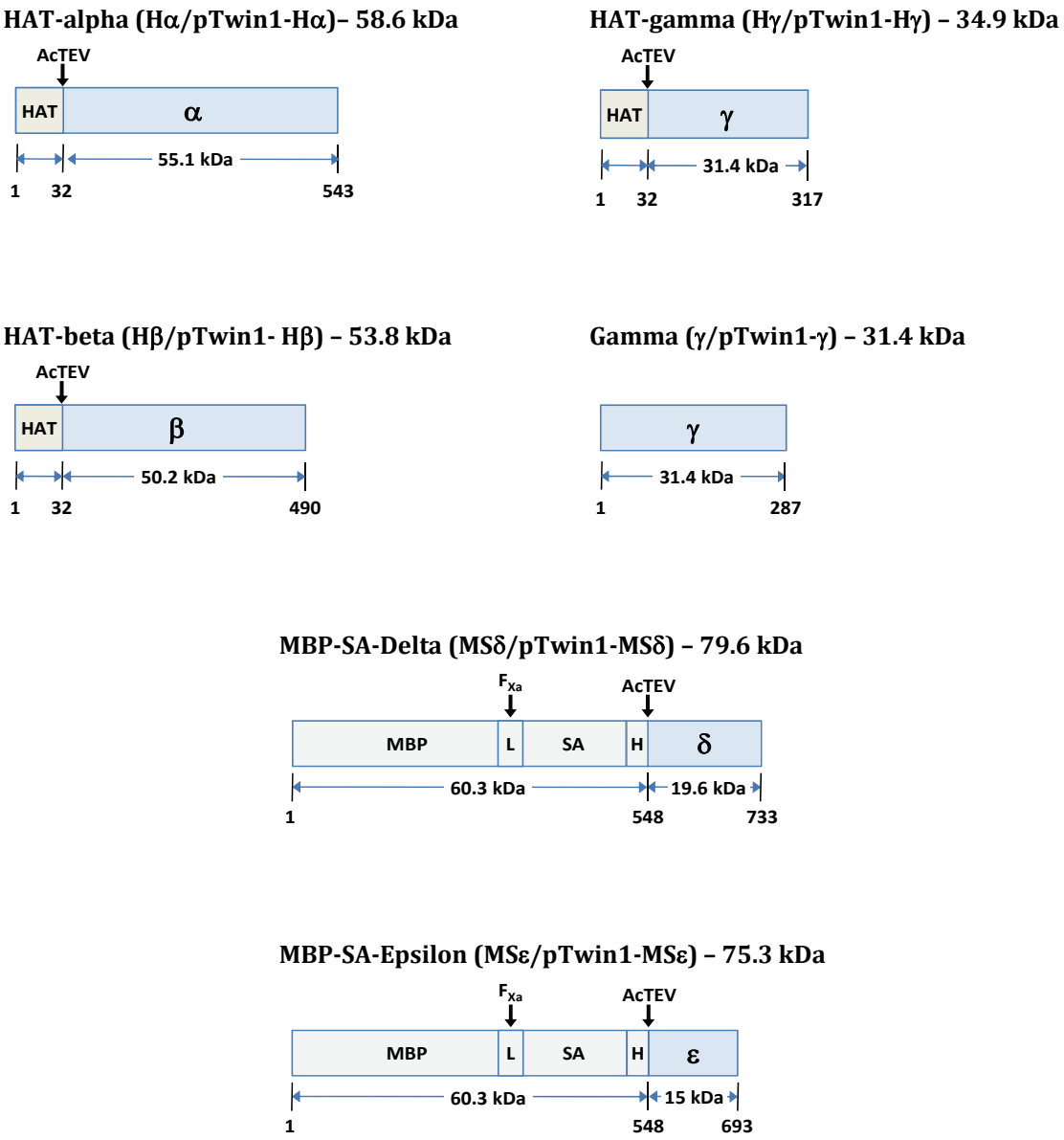

Figure 4.4: Schematic overview of the recombinant ATPase subunits produced in this study. Abbreviations: AcTEV = Modified Tobacco Etch virus protease cleavage site, MBP = Maltose binding protein (38.6 kDa), H = 6xHis tag, L1 = Linker with factor Xa protease cleavage site (F_{Xa}), HAT = Histidine Affinity Tag (3.3 kDa) and SA = Streptavidin (17.8 kDa). The numbers under the figures reflect the residue position in the primary sequence. The calculated molecular weight is noted in the legend above the individual subunits.

4.3 Preliminary studies on the over expression of ATPase subunits

4.3.1 Identification of conditions for subunit over expression

Tests were conducted to investigate the best temperature for over expression of the recombinant subunits. In each case the test was carried out in 10 ml shaken test tube cultures using LB medium supplemented with ampicillin, which had been inoculated with 0.2 ml of an overnight preculture. To increase biomass before induction, the cultures were incubated at 37°C for 4 hours before being placed at the temperature at which expression was tested. They were then induced using 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubated for 3, 5 or 8 hours. The cells were then harvested and lysed using lysozyme (0.5 mg/ml for 30 minutes at room temperature), and subsequently spun at 5000 rpm for 10 minutes in a microfuge (Ole Dich, type 154.RF). The resulting pellet was resuspended in denaturation buffer and incubated for 30 minutes at room temperature. After disruption of the cells, debris was removed by centrifugation (17000 rpm for 5 minutes in a microfuge) and a sample of the resulting supernatant analysed using SDS-PAGE (Figure 4.5). The investigation revealed that the heaviest band for the individual subunits on the gels was found after incubation of the cells at 37°C and that this temperature therefore gave the highest level of expression (see Figure 4.5).

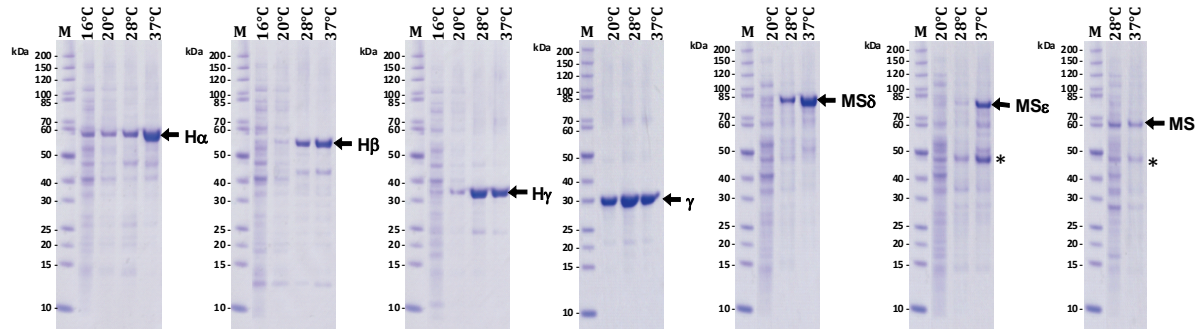

Figure 4.5: Reduced SDS-PAGE gel illustrating the impact of temperature on the over expression of the recombinant ATP synthase subunits. The growth temperature is noted at the top of each lane and the arrow indicates the position of the expressed protein of interest. Cells cultivated at 16°C were incubated overnight, while cells grown at 20°C, 28°C and 37°C were incubated for 8, 5 and 3 hours, respectively, after addition of the 0.5 mM IPTG. The total protein content of each lane was adjusted to 5 µg in total. The gel furthest to the right shows the result of induction of the cells carrying the original pET15b-MBP-SA plasmid (MS: 59.3 kDa). The theoretically calculated molecular sizes are: Hα: 58.6 kDa, Hβ: 53.6 kDa, Hγ: 34.6 kDa, γ: 31.8 kDa, MSδ: 79.6 kDa and MSε: 75.3 kDa.

4.3.2 Large scale culture handling

A freshly transformed colony carrying the expression plasmid in question was transferred from a selective solid LB medium to 10 ml liquid LB medium supplemented with 100 µl/ml ampicillin (LB+amp) and incubated overnight at 37°C with shaking. The resulting preculture was used to inoculate a 2 L Erlenmeyer flask containing 1 L freshly prepared LB+amp media, which was afterwards incubated at 37°C with shaking until the optical density (OD at 600 nm) reached 0.4-0.8. At this point the transcription and thus translation of the recombinant ATP synthase unit was initiated by addition of 0.5 mM IPTG. Cultivation was continued at 37°C for an additional period of 2 hours after which the biomass was recovered by centrifugation at 5000 rpm for 10 minutes. The wet biomass (approximately 2-3 g) was subsequently stored at -20°C until needed.

4.3.3 Solubilization characteristics of the recombinant ATPase subunits expressed

It was suspected that the subunits produced in the large scale cultures were present as both a correctly folded soluble fraction and as inclusion bodies within the host cells. To determine the best solubilization conditions for the over expressed subunits resulting from the large scale cultures, 5 ml samples were spun at 5000 rpm in a microfuge and the resulting cell pellet resuspended in 2 ml PBS buffer supplemented with 1 mg/ml lysozyme and 1 mM PMSF, followed by a 30 minute incubation period at room temperature. After lysis was performed, the suspension was spun at 17000 rpm for 5 minutes and the supernatant removed (referred to as the PBS fraction). The pellet was washed three times using 50 mM sodium phosphate buffer (pH 8) before being split up in equal sized fractions. The separated fractions were subsequently treated with various concentrations of urea to dissolve any inclusion bodies and incubated at 4°C overnight, after which they were spun at 17000 rpm for 10 minutes. The resulting supernatant were collected (designated as the S fraction), while the remaining pellet was washed 2 times before being dissolved in 8 M urea supplemented with 1% Triton-X and 10 mM mecaptoethanol (referred to as P fraction). The collected samples were analysed by SDS-PAGE (Figure 4.6). For cells overexpressing the native γ subunit or either of the maltose-streptavidin constructs (MSδ/MSε) only the PBS as well as the urea supernatant fractions were analyzed. The results (Figure 4.6) show that of all the subunits, only H□ had a large band present in the PBS fraction, indicating that only this

subunit was soluble. All the other subunits required treatment of the cell pellet with urea to solubilise the protein, indicating that they were primarily present as inclusion bodies. For all subunits, considerable solubilisation was possible by using 2-3 M urea, but the maximum yield was found using 8 M urea.

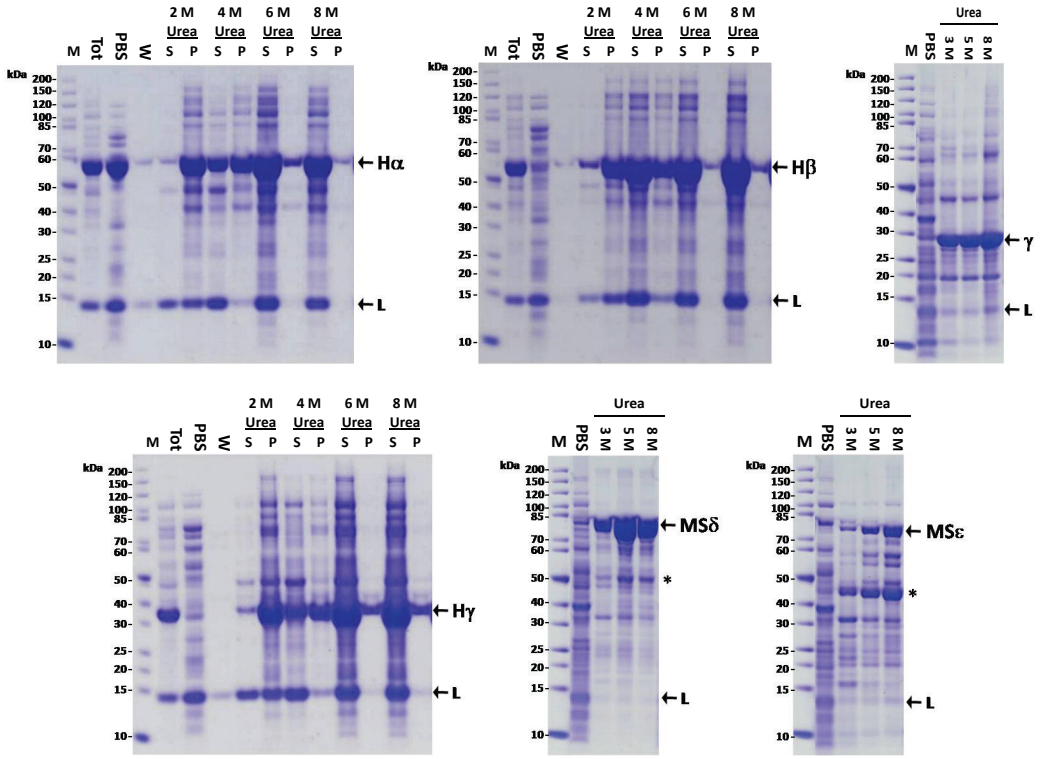

Figure 4.6: Reduced SDS-PAGE gels showing the proteins released after treatment of samples from theATPase subunit over expressing strains. The labels at the top of the gels refer to a sample treated in the following way: *Tot* = untreated sample obtained after lysis, PBS = soluble in PBS, W = pooled fractions from wash of pellet from lysed cells, S = solubilised in the noted urea concentration, P = insolubilized fraction in the noted urea concentration. The arrow indicates the position of the band corresponding to the expected size of the expressed recombinant subunit in question. Expected (theoretical) sizes were Hα: 58.6 kDa, Hβ: 53.6 kDa, Hγ: 34.6 kDa, γ: 31.7 kDa, MSδ: 79.6 kDa and MSε: 75.3 kDa. 'L' shows the position of the added lysozyme and ' * ' shows what is believed to be pre-terminated recombinant products, while 'm' is a lane containing protein standards.

4.3.4 Purification of the over expressed subunits

In the section above, it was established by SDS-PAGE analysis that only the HAT-alpha subunit could be recovered directly from the cell lysate in a soluble form in PBS buffer. The remaining recombinant subunits appeared to be expressed as inclusion bodies (see Figure 4.6). Based on this observation it was decided to conduct the purification process using a phosphate buffer supplemented with 8 M urea to achieve the highest possible yields. The HAT affinity tag is known to function in denatured proteins. All polyhisitidine tagged subunits were pretreated using a common approach, which involved cell disruption using Cell Lytic B reagents (Sigma-Aldrich) supplemented with 0.5 mg/ml lyzosyme and 1 mM phenylmethanesulphonylfluoride (PMSF) as described by the manufacturer. The cell debris was subsequently pelleted by centrifugation at 17000 rpm for 10 minutes at 4°C in a microfuge, washed three times times using PBS buffer and finally resuspended in 80 ml solubilization buffer (8 M urea, 20 mM Tris-HCl, pH 8).

Immobilized metal ion affinity chromatography (IMAC) was performed using a Gradifrac and a peristaltic pump (P-1), with a C16/20 column packed with 20 ml of chelating Sepharose Fast flow matrix charged with Ni^{2+} according to the manufacturers recommendations (all products of Amersham Biosciences AB, SE-751 84, Uppsala, Sweden). To monitor the outlet of the column, a UV-detector and a pH-detector were used between the column exit and the fraction collector. For each of the recombinant ATPase subunits, the metal affinity chromatography purification was performed using a similar approach. 65 ml of the solubilized subunit in question was loaded on to the column at 1 ml/min followed by a washing step (50 mM Na_2HPO_4, pH 5.9, 8 M urea) conducted at 2 ml/min until no protein was detected by the UV monitor. Finally the bound protein was eluted from the column using an elution buffer (100 mM Na_2HPO_4, 8 M urea, 20 mM Tris-HCl, pH 4.5) at 1 ml/min. After elution, the column was regenerated by washing with a stripping buffer (20 mM Na_2HPO_4, 50 mM EDTA, pH 7.2). As soon as the purification cycle was completed the collected samples were analyzed for total protein using the BCA assay. The fractions containing the eluted protein were identified, pooled together and the pH was adjusted to 7.4 using NaOH. The procedures lead to the collection of between 3-6 mg/ml corresponding to a total of 8-12 mg protein, depending on the subunit in question. In case of over expressed native γ subunit (i.e. without the HAT-tag) the pellet from cell lysis was washed three times in PBS followed by two

washing steps using a 50 mM phosphate buffer (pH 8) supplemented with 3 M urea. The remaining pellet containing the γ subunits was finally dissolved in solubilization buffer (i.e. containing 8 M urea) overnight at 4°C. The H□, H□, H□ and □ subunits were estimated by visual inspection of SDS-PAGE gels to have purities greater than 90% (Figure 4.7).

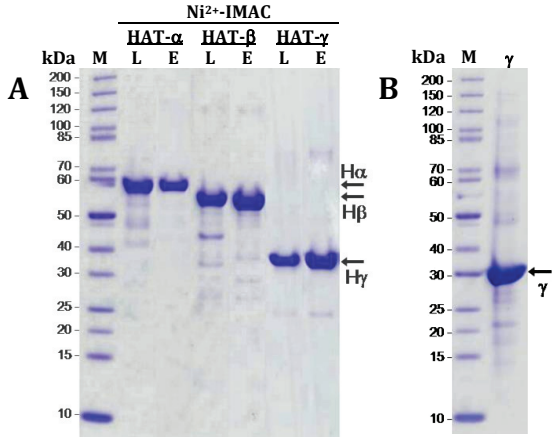

Figure 4.7: Reduced SDS-PAGE analysis of the subunits after purification: (A) The HAT-α, HAT-β and HAT-γ. **(B)** γ-subunits from washed inclusion bodies. 'L' Indicates the loaded sample and 'E' is that eluted. 'M' Refers to protein standards.

SDS-PAGE analysis of the IMAC purified MSδ and MSε recombinant subunits (Figure 4.8) revealed that the samples contained signification amounts of impurities and to improve the purity, an additional step of gel filtration using Sephacryl S-300 HR (GE Healthcare Bio-Sciences AB) was performed. A 60 cm long column with a 0.9 cm diameter was packed with 38 ml gel matrix and 2 ml of sample was loaded onto the column at 0.2 ml/min, followed by continued pumping of solubilization buffer at the same speed. The contents of all collected samples were analyzed by SDS-PAGE and the results revealed that the gel filtration step had significantly improved the purity of the MSδ subunit, while the procedure had been less successful in the case of the MSε. Supplementary material including extended SDS PAGE analysis of the collected fractions and chromatograms may be found in appendix C.

To summarise, for the studies of ATPase assembly on magnetic adsorbents, the H□, H□ and H□ subunits were purified by IMAC, the □ by inclusion body washing and solubilisation only, the MBP-SA-□ by IMAC and SEC and the MBP-SA-□ by IMAC.

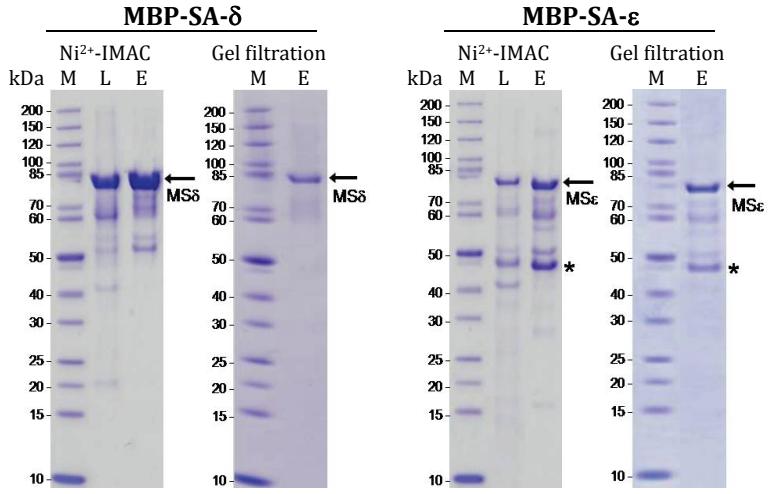

Figure 4.8: Reduced SDS-PAGE analysis of the purification of the recombinant MSδ and MSε. Purification was performed using Ni^{2+} charged Sepharose Fast flow gel (IMAC) and gel filtration (Sepharose S-300 HR). '*' Refers to a band that is believed to originate from pre-terminated translation of the recombinant MSε. 'L' Indicats the loaded sample and 'E' what is eluted. 'M' = protein standards.

4.4 Results and discussion

To ensure the greatest chance of succeeding in the assembly and reconstruction of an active form of the F_1-ATP synthase complex using magnetic supports, three main strategies were planned based on the use of three different types of magnetic support. However, in all cases the methods employed were inspired by successful previous reports by Futai (1977), Dunn et al. (1979), Shin et al. (1996) and Ekuni et al. (1998). Before attempting assembly on the magnetic supports, it was verified that active and thus correctly reconstructed F_1-ATPase could be prepared in solution from the purified denatured subunits produced above. A mixture of the denatured Hα, Hβ, Hγ/γ and MSδ/ MSε were mixed in various combinations using a stoichiometric molar ratio of ca. 3:3:1:1 (10 µg, 9 µg, 2 µg and 2 µg) and dialyzed overnight at room temperature against a reconstruction buffer consisting of 50 mM succinate-tris, pH 6 supplemented with 5 mM ATP, 5 mM $MgSO_4$, 10% w/v glycerol, 1 mM DTT and 0.5 mM EDTA. To establish that active F_1-ATPase was formed the resulting dialyzed samples were centrifuged (13000 rpm for 10 minutes), the supernatant recovered and analysed for protein and ATP hydrolysis activity.

The results in Figure 4.9 show the rate of inorganic phosphate production from ATP for different combinations of subunits. For all combinations, ATP hydrolysis was observed, indicating active assembled ATPase was produced. The highest activity was seen from the combination of H□ + H□ + □ subunits. Furthermore it was possible to achieve assembly using both the native form of the γ subunit as well as Hγ. The results (Figure 4.9) confirm previously published activity data by Shin et al. (1996), who reported that complexes formed with δ or ε exhibited high and low activity respectively. The results also show that greater activity was found when the □ rather than H□ subunit was used for assembly (Figure 4.9). Complexes formed in the absence of MSδ and MSε displayed the highest activity when the γ subunit was used and an activity comparable with MSε-containing complexes when Hγ was included.

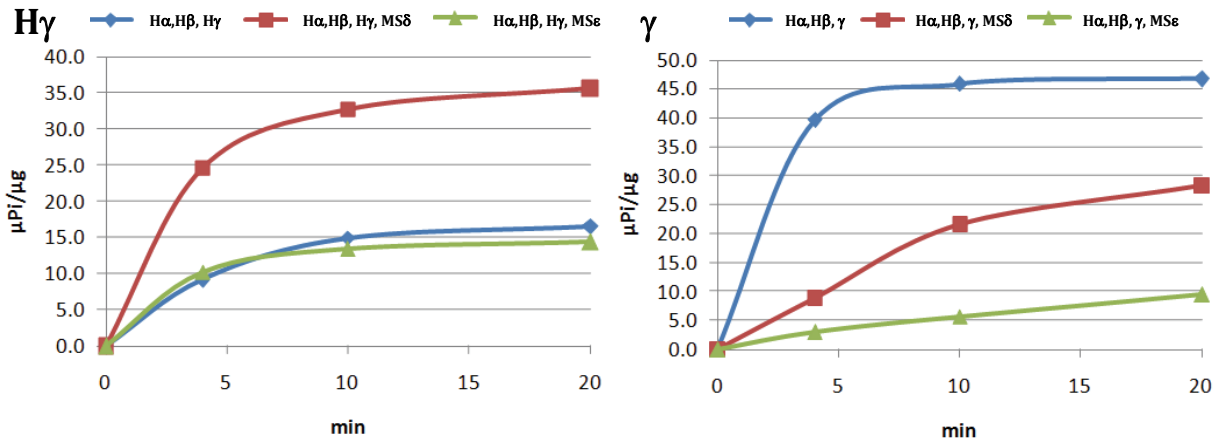

Figure 4.9: Rate of inorganic phosphate production from ATP per gram of protein by the ATPase assembled from different combinations of subunits. Left figure shows activity measurements of F_1-ATPase prepared using the Hγ while the right graph shows activity results obtained using a native γ-subunit. All data points were normalized by subtracting the corresponding background hydrolysis (no protein).

4.4.1 Assembly of the F_1-ATP synthase using IDA coupled magnetic particles

The simplest strategy possible was investigated in the first attempt of assembly of the F1-ATPase on magnetic supports. This approach involved anchoring the central gamma subunit (see Figure 4.1) to the support in the first step and adding the and subunits in a subsequent step, which should then self assemble to provide active ATPase (Figure 4.10). The straight forwardness of the approach employing the minimal subset of subunits known to give an active complex (Futai et al., 1977) was expected to outweigh the above finding (Figure 4.9) that non-tagged subunit resulted in the highest activity. Iminodiacetic acid (IDA) coupled non-porous polyglutaraldehyde magnetic particles (IDA-mPGA) prepared as described in the materials and methods were employed. The magnetic supports were first charged with copper(II) ions followed by initial binding of the purified HAT tagged γ subunit (Hγ) under denaturing conditions. The next step involved the addition of the remaining denatured subunits in the appropriate stoichimetric ratios followed by a 60-fold rapid dilution of the urea to promote refolding and reconstitution of the complex around the immobilized Hγ-subunit. Subsequently, the formed complex was eluted using imidazole (Figure 4.10).

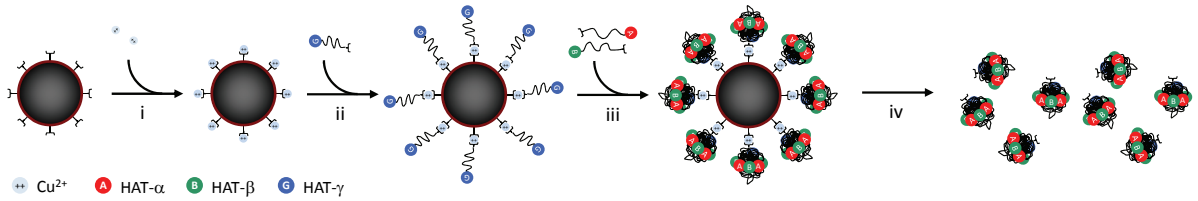

Figure 4.10: Schematic of the approach to assemble F1-ATPase using Cu^{2+} charged IDA derivatized magnetic supports. The approach involved a simple four step process: (i) Copper charging, (ii) Capture of the Hγ subunits, (iii) Addition of Hα and Hβ and rapid dilution of the urea (60x) and (iv) Elution of bound complexes using 250 mM imidazole.

To study the performance of Cu^{2+} charged IDA coupled magnetic support, an investigation of the adsorption of the purified, denatured HAT-tagged γ subunit in 8 M urea was performed with the aim of verifying Langmuir type binding characteristics (Figure 4.11).

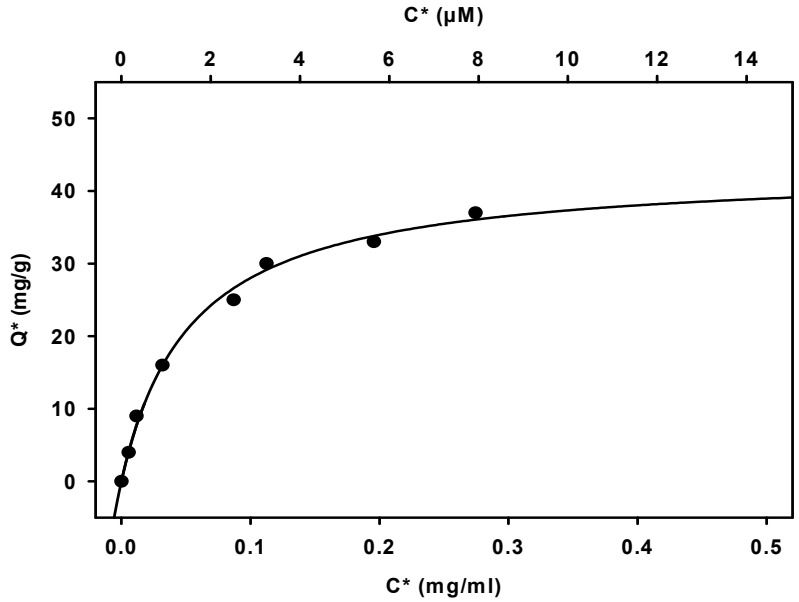

Figure 4.11: Equilibrium binding isotherm for denatured HAT-γ using copper(II) charged IDA-mPGA particles. Data points were obtained by measurement of the unbound protein fraction using the BCA assay and fitted to the Langmuir equation.

The data in Figure 4.11 shows a typical Langmuir type adsorption isotherm displaying a steep initial slope followed by a plateau. The data could be fitted with the Langmuir model and the calculated maximum binding capacity (Q_{Max}) and dissociation constant (k_d) were found to be 43.3 mg/g and 1.56 μM, respectively, which demonstrate good binding. The Q_{Max} value is similar (43.9 mg/g) to that of Ferré (2005), who used the

same type of adsorbents for binding an unfolded recombinant HAT tagged protein (human □2m) of similar size (29 kDa versus 34.9 kDa for H□). However, in that work, a value of 0.038 μM was reported for k_d .

Despite the promising adsorption isotherm for H□ a problem connected with the use of the IDA-coupled support for reconstitution of the F_1-ATPase was identified. In previously published reports by Futai et al (1977), Dunn et al. (1980) and Shin et al. (1996) and others, the buffer used during assembly was supplemented with both dithiothreitol (1 mM DTT) and ethylenediaminetetraacetic acid (0.5 mM EDTA). EDTA will be expected to chelate the copper ion on the magnetic support, thus releasing affinity bound proteins and DTT will reduce the Cu(II) on the magnetic supports to Cu(I) thus also releasing bound proteins. In addition to this the pH value of 6, which was shown by previous workers to promote the highest degree of ATPase assembly is too low to maintain adequate binding to the metal chelate support due to the pK_a of the histidine residues in the HAT-tag (Terpe et al. 2003). The incompatibility of 1 mM DTT was confirmed by observing that it's addition to a solution of 0.1 M $CuSO_4$ caused a colour change from blue to green, i.e. from copper(II) to copper(I). To investigate the impact of buffers with 0.5 mM EDTA or pH 6 on adsorption, an investigation of the release of Hγ from Cu^{2+} charged magnetic supports was performed and the results analysed by SDS-PAGE. The results in Figure 4.12 show that no band was seen (lanes 2 and 3) in supernatants following two consecutive 5 minute washes of H□ preloaded magnetic supports with buffers containing 0.5 mM EDTA and then Tris-succinate at pH6. The elution fraction (lane 4) contained a single clear band demonstrating that the presence of 0.5 mM EDTA or use of pH 6 had little negative effect on H□□binding.

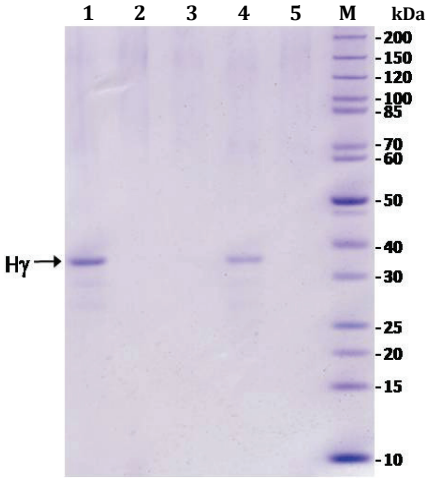

Figure 4.12: Reduced SDS-PAGE gel showing the effects of buffers containing 0.5 mM EDTA or at pH 6 on the release of H bound to copper(II) charged IDA derivatized magnetic supports. Lane (1) contains a sample of the loaded H, (2) EDTA wash fraction (0.5 mM) and (3) succinate-Tris wash fraction (pH 6). Lanes marked (4) and (5) contain consecutive elution samples obtained after 10 minutes incubation using a buffer supplemented with 250 mM imidazole. The size of the recombinant Hγ is 34.6 kDa and the corresponding band is marked with an arrow. 'M' Refers to a protein standard. Adsorption was performed for 30 minutes using 4 mg Cu^{2+} charged magnetic support suspended in 1 ml 100 mM sodium phosphate buffer/8 M urea (pH 8) containing 0.6 mg/ml Hγ. Washing and elution was in each case performed for 5 minutes in 0.4 ml buffer. All buffers contained 8 M urea.

It was thus concluded that 0.5 mM EDTA as well as buffers at pH 6 were compatible with the strategy of employing IDA magnetic supports for reversible attachment of the first subunit in the process of ATPase assembly.

To further explore the assembly strategy, the next step in the investigation was to add the remaining subunits in urea, followed by changing the conditions from denaturing to native, by rapid dilution. Thus the magnetic supports were charged with Cu(II) ions, then H in 8 M urea containing buffer was attached and washed in buffer, also containing urea, before both subunits in urea were added and the mixture diluted 60 times in modified assembly buffer and incubated overnight at room temperature under rotation. After washing, the supports were eluted with imidazole and the samples analysed by SDS-PAGE. The analysis showed no bands corresponding to any of the ATPase subunits in the elution fraction (Figure 4.13 lane 3), suggesting that assembly was unsuccessful. Attempts to measure ATP hydrolyzing activity of the collected wash fractions were abandoned due to undetectable amounts of protein (below 0.01 mg/ml). Figure 4.13 lane 2 shows that all subunits were present during the overnight incubation of the mixture; as expected the H band is barely visible since it should not be released from the adsorbent and the H and H are present in excess.

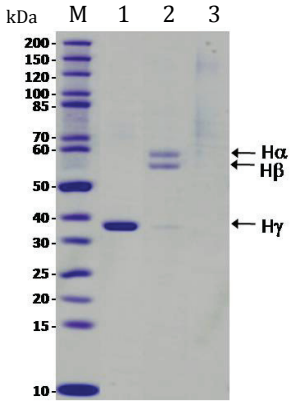

Figure 4.13: SDS-PAGE gel showing the results of a study of the assembly of the F_1-ATP synthase using the recombinant HAT tagged subunits. Assembly was performed as described and involving capture of Hγ under denaturing conditions, followed by addition of Hα and Hβ and rapid dilution converting from denative to native conditions (60x dilution). The lane marked (1) contains a sample of the Hγ containing fraction used during the initial adsorption step, while the lane marked (2) contains a acetone precipitated sample secured after incubation with Hα and Hβ, while the lane designated (3) shows the elution sample (acetone precipitated). The arrows indicate the identity of the bands and (M) refers to a standard protein marker.

The lack of evidence of elution of any of the subunits, combined with the presence of the faint band for H□ in lane 2 (Figure 4.13) may indicate that the long overnight incubation at a pH not optimal for binding, and in dilute solution, pushed the isotherm for H□ binding (Figure 4.11) far to the left as the system approached a new binding equilibrium. This would leave very low amounts of assembled ATPase on the supports for detection following elution. Alternatively, it is possible that the shift from denaturing to native folding conditions and the resulting conformational change, in the subunits may have resulted in premature release from the magnetic support. Given the potential problems with the metal chelate affinity immobilisation, a new strategy was examined for ATPase assembly on magnetic supports.

4.4.2 Reconstruction of the F_1-ATPase using amylose magnetic beads

The second strategy employed for assembly of the F_1-ATP synthase was based on the use of commercially available amylose coupled agarose-based magnetic beads (New England Biolabs) and subunits with the Maltose Binding Protein tag (MBP). The strategy resembled the previous approach (see Figure 4.10) used with the IDA-linked support and involved a series of steps, which revolved around preliminary adsorption of either the δ (MSδ) or the ε (MSε) subunits via the MBP tag followed by addition of the remaining components (Hα, Hβ and Hγ/γ) (Figure 4.14). However, unlike the previous study in which the first binding step was performed in a denaturing environment, the initial step of adsorption in this case needed to be carried out under native conditions to ensure that the 38.6 kDa MBP affinity tag was correctly folded. To achieve desorption from the supports the MBP tag allows elution using a maltose supplemented buffer. Given that MS□ and MS□ were produced as inclusion bodies and purified in 8 M urea, it was necessary to dialyse overnight against MBP binding buffer (20 mM tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4) using a 12.4 kDa cut off cellulose based tube to permit refolding.

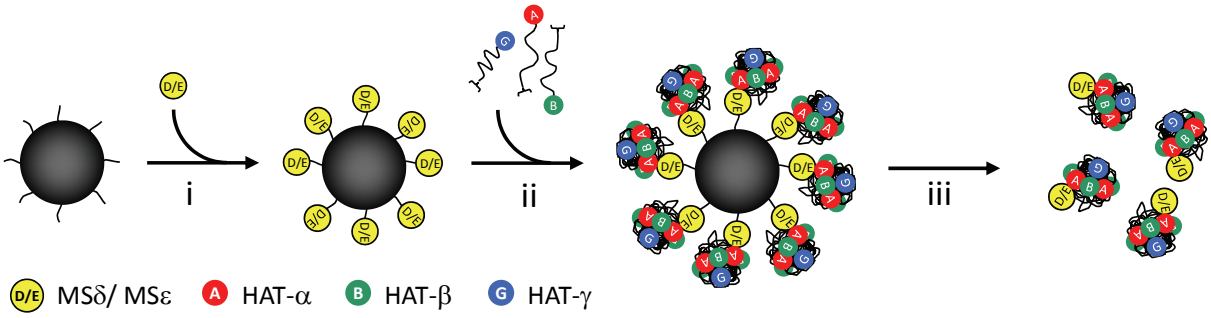

Figure 4.14: Schematic of the assembly strategy using amylose magnetic beads. (i) Adsorption of native MSδ or MSε, (ii) Addition of Hα, Hβ, Hγ and dilution to allow refolding and assembly, and (iii) Elution of the complexes formed using maltose.

After the dialysis and thus the change from denaturing to native conditions, the suspensions containing MSδ or MSε were found to be turbid and thus were centrifuged (13000 rpm for 5 minutes) to obtain a clarified solution. The recovered supernatants were found to contain 0.3 mg/ml protein for the case of both MSδ and MSε, which was presumed to be the maximum obtainable concentration of each subunit under the refolding conditions used.

To investigate if dialysis had led to correctly folded MBP tags a simple binding study was performed. For each of the subunits (MSδ and MSε), 10 mg amylose magnetic beads were prepared by three serial washing steps performed using MBP binding buffer followed by an incubation step with 500 µl dialyzed MSδ and MSε containing (each containing 0.3 mg/ml) solutions for 1 hour at 4°C with gentle mixing by rotation. Subsequently the magnetic beads were recovered by magnetic separation and washed three times in 1 ml MBP buffer to remove excess unbound protein. The bound protein was released using 100 µl MBP elution buffer (MBP buffer supplemented with 10 mM maltose) with a 10 minute incubation under rotation.

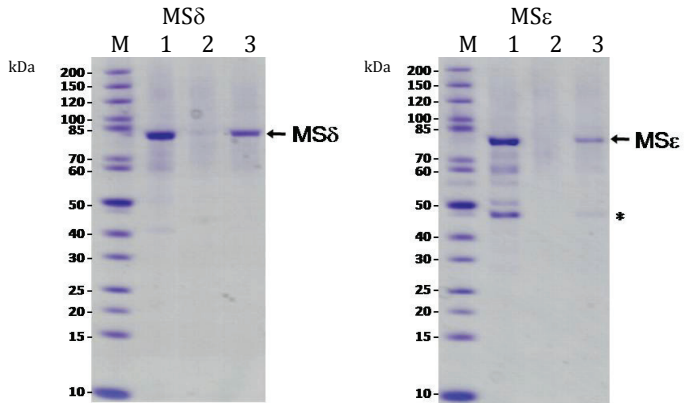

Figure 4.15: Reduced SDS-PAGE gel analysis of fractions obtained during binding studies of MBP tagged MSδ and MSε subunits using magnetic amylose beads. Lanes marked (1) contain the solution used for adsorption, (2) wash fraction and (3) the eluted fraction. '*' Refers to a band that is believed to originate from pre-terminated translation of the recombinant MSε.

SDS-PAGE analysis of the elution fraction (Figure 4.15, lane 3 in both gels) showed bands corresponding to MS□ or MS□, demonstrating that the refolded subunits could be captured, retained and released from the magnetic supports as expected. The gels also suggest that the capacity of the magnetic amylose beads was dependent on the subunit; MS□ had a greater band intensity in the eluted samples as compared to MS□ (in both cases the same protein concentrations were used for support loading). Measurements of the eluted protein content verified this finding: Approximately 1 µg MSδ could be eluted from 10 mg support, while only 0.6 µg were recovered from the same amount of particles in the case of the MSε subunit. These values correspond to a capacity of the amylose coupled magnetic support of 10 mg/g and 6 mg/g, respectively. The difference in apparent capacity/elution efficiency was to some extent surprising as the subunits were almost equal in size and had the same N-terminal sequences. A possible

explanation for the differences in capacities may be the presence of the impurities in the MS solution, which could not be removed using both affinity chromatography and gel filtration (Figure 4.8). The fact that the procedure used during the purification process had failed to remove the impurities indicated a physical interaction with the MSε subunit, and this interaction may have had an impact on the binding of the subunit. For more details on the purification procedure see appendix C.

Based on the successful results obtained in the study of initial adsorption of the MSδ and MSε, the remaining strategy aimed at achieving ATP synthase reconstruction was performed. Fresh amylose coupled magnetic supports (40 mg) were prepared as previously described and split into two, then incubated with 1 ml of dialyzed solution containing either the MSδ or the MSε subunit with mixing by rotation for 1 hour at 4°C. After a washing step performed using MBP buffer (5 minutes under rotation), the supports were distributed into 4 mg aliquots, separated and resuspended in 400 µl assembly buffer. To each of the aliquots, various combinations of the other subunits (see Figure 4.16) were subsequently added, to test the ability to bind these (i.e. to assemble the F1 ATPase). The various combinations of subunits were added in the stoichimetric ratio 3:3:1 (Hα, Hβ, Hγ/γ) or more specifically using 8 µg, 7 µg and 1.5 µg, respectively. All mixtures were incubated under rotation at room temperature overnight and subsequently washed three times in assembly buffer to remove unbound material before being incubated in 200 µl MBP elution buffer.In addition to this, a control was conducted in which 12 mg fresh support was also split into three tubes, resuspended in 400 µl assembly buffer and various combinations of Hα, Hβ and Hγ/γ added to investigate the ability to bind these subunits without the presence of pre-adsorbed MSδ and MSε. In all cases the total volume of added subunits were kept under 5 µl to ensure at that the dilution of the urea was at least 80-fold resulting in a decrease from 8 M to 0.1 M urea.

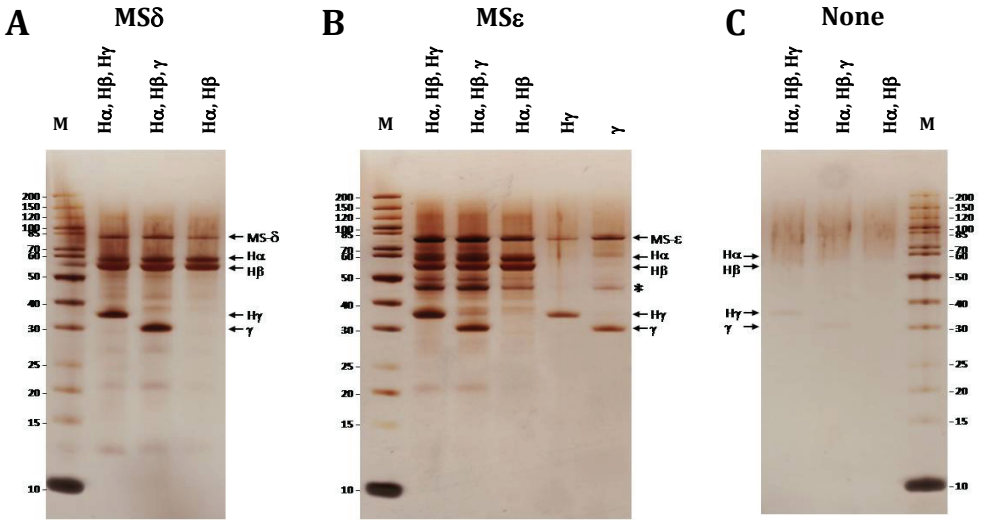

Figure 4.16: Silver stained reduced SDS-PAGE showing the assembly of F_1-ATPase using magnetic amylose beads. All samples were acetone precipitated and up concentrated 10 times before the analysis. (A) Pre-adsorption of the MSδ adsorption, (B) Pre-adsorption of the MSε adsorption and (C) no pre-adsorption of MSδ/MSε performed. (M) Refers to a protein maker. The Arrows indicate the identity of each band, while indicates a known MSε related impurity.

The results showed that if MSδ was attached to the magnetic adsorbents, then combinations of Hα, Hβ or Hα, Hβ, Hγ/γ could be recovered in elution fractions (Figure 4.16 A) which is highly suggestive of an interaction with the immobilised MS and ATP synthase assembly when all four subunits were present. The same results could be observed when MSε was preloaded to the adsorbents (Figure 4.16 B) and in addition to this, Hγ and γ could also be captured in the absence of Hα and Hβ. The contention that assembly had occurred is supported by the control experiment in which no elution of H, H, H or was seen if MS or MS were not preloaded onto magnetic adsorbents (Figure 4.16 C). The results in Figure 4.16 also indicated that both Hα or Hβ could be captured using magnetic supports preloaded with MSδ or MSε, and this result is in full agreement with a previous study by Aris and Simoni (1983), who found a similar interaction using cross-linking analysis. Interaction between the δ subunit α and β subunits was also demonstrated using two hybrid interaction analysis by Moritani et al. (1996), however in that study no interaction between ε and α or β could be established. The conclusion based on the SDS-PAGE analysis (Figure 4.16) suggested that the strategy of using the MBP tag combined with the amylose coupled magnetic support successfully resulted in assembly. However to verify that the reconstruction was correct

and that active F_1-ATPase was formed, the ATP hydrolyzing activity of the eluted samples was examined as described above. Despite repeated attempts to measure activity in the eluted samples none could found, which is believed to be due to the very low amounts of the ATPase, as shown by measurement of the protein concentration (<0.02 mg/ml). It was concluded that a more sensitive activity assay was needed. For this reason HPLC analysis of the samples' ability to produce ADP from ATP was also performed as described in the materials and methods. However this approach also failed to give evidence supporting ATP hydrolysis mediated by the reconstructed F_1-ATP synthase and for this reason it could not be confirmed that the assembled complexes were functional.

4.4.3 Assembly mediated by biotin derivatized magnetic supports

In the above two strategies for ATP assembly, evidence was seen from SDS-PAGE analysis that F_1-ATPase assembly had occurred on the magnetic supports but in both cases ATP hydrolysing activity could not be demonstrated. A major cause of this was believed to be that the amount of F_1-ATPase assembled was too low for activity to be measured. Part of the reason for this could be the affinity tags used which were chosen for their easily reversed binding (to permit elution), but which may thus have allowed dissociation and loss of complexes during assembling and wash steps. Thus a more robust attachment method was sought. The biotin-streptavidin interaction is essentially permanent but the inclusion of an enzymatic cleavage site can be used to allow release from a support, and this system was chosen as the third strategy for ATPase assembly on magnetic supports.

Biotin derivatized polyvinyl alcohol based magnetic beads were employed which could be used to immobilise the δ and ε recombinant subunits, which possessed a streptavidin domain following the maltose binding domain (Figure 4.3). The general approach resembled the two previously described strategies and consisted of a sequence of steps, starting with pre-absorption of the streptavidin domain cotaining MSδ or MSε to the magnetic support, followed by addition of the remaining subunits (Figure 4.17). Due to the extraordinarily strong binding between biotin and the streptavidin (Gitlin et al., 1988), a final step involving proteolytic release of the assembled ATPase from the support mediated by the AcTEV protease was used. In the event that protease mediated release of the complexes was not efficient, complete denaturation using denaturation buffer supplemented with 0.1% the non-ionic surfactant Triton-X 100 was employed (Figure 4.17).

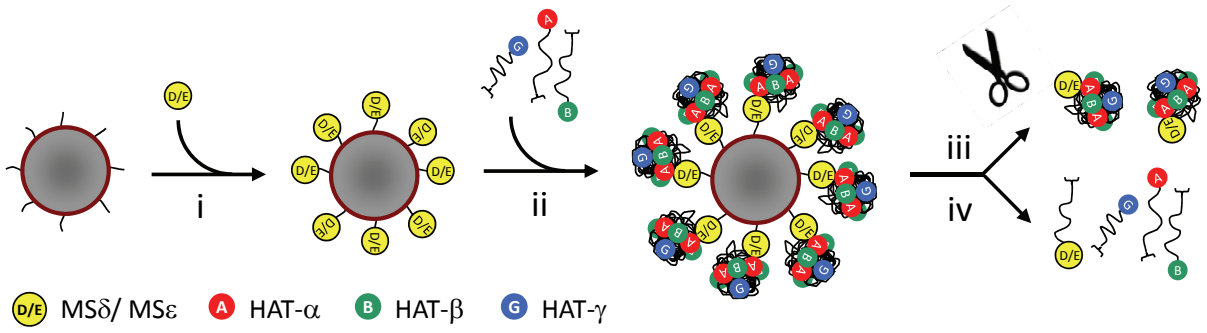

Figure 4.17: Schematic of the approach to ATPase assembly using biotin derivatized magnetic supports. (i) Adsorption of MSδ or MSε under native conditions, (ii) Addition of Hα, Hβ, Hγ and dilution to native conditions, (iii) Elution of complexes formed by proteolytic digestion or (iv) release by denaturation.

Before any binding studies were performed, attention was directed towards the AcTEV sites inserted in all the recombinant subunits and the ability of the AcTEV protease to successfully mediate the necessary release of the formed complex from the magnetic support. Given that the subunits were purified in a denatured state, urea was first removed from them by dialysis overnight at room temperature using a 12.4 kDa cut-off membrane against a 50 mM Tris-HCl buffer, pH 8 supplemented with 0.5 mM EDTA and 1 mM DTT. As previously observed, a turbid solution resulted which was clarified by centrifugation. Digestion with AcTEV protease was then conducted using 500 µl of clarified dialyzed sample overnight at 4°C using the recommended dosage of 0.5 U of activity per 1 µg protein. However, a subsequent SDS-PAGE analysis revealed that the digestion was not successful and it was also observed that the incubation had resulted in a turbid solution indicating that protein aggregates had formed. To improve the performance of the AcTEV protease, the experiment was repeated at room temperature using a 5-fold higher concentration of activity (2.5 U/µg). Visual inspection of the solution after the overnight digestion indicated that aggregate formation was reduced but not eliminated. Thus prior to SDS-PAGE analysis, the incubated samples were clarified by centrifugation and then concentrated 10-fold by acetone precipitation. The results are shown in Figure 4.18.

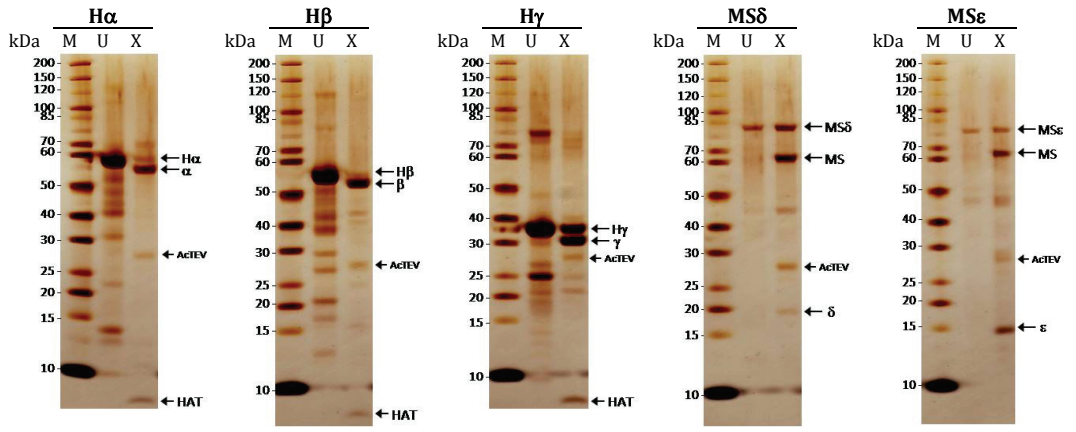

Figure 4.18: Silver stained reduced SDS-PAGE illustrating the effects of AcTEV proteolytic digestion of the recombinant subunits performed at room temperature. The digest was carried out using a 5-fold higher concentration of the AcTEV compared to that recommended. The type of subunit is noted at the top of each gel. U refers to an untreated sample, while X is digested with the AcTEV protease overnight at room temperature. The arrows indicate the identity of the corresponding bands.

Based on the results shown in Figure 4.18 it could be seen that the proteolytic digest performed at room temperate resulted in an almost complete removal of the HAT-tag in the case of the Hα and Hβ. In both cases the H band almost completely disappeared, being replaced by a slightly lower molecular weight species and a band below 10 kDa, corresponding to the HAT tag. However in the case of MSδ and MSε, and especially the Hγ subunit, only partial digestion was seen, despite the addition of 5-fold more proteolytic activity than recommended and prolonged overnight incubation at room temperature. A possible and very likely explanation for the less successful performance in the case of the Hγ and MSδ or MSε may be that the AcTEV site was only partially accessible to the protease due to the folding of the subunits. However, the investigation did demonstrate that all subunits could, to some extent, be cleaved by the site specific AcTEV protease.

As described in the previous strategy (i.e. with the use of the amylose magnetic beads), the first step in the analysis of the assembly of the F_1 ATP synthase was to verify the ability of refolded subunits to be captured by the biotin-derivatized magnetic support. For this reason 500 µl of a 0.3 mg/ml solution of MSδ or MSε (clarified and dialyzed against MBP buffer) was incubated with vigorous shaking for 1 hour at room temperature together with 2 mg biotinylated mPVA support. After the incubation period

the support was washed 3 times in fresh MBP buffer for 5 minutes with shaking and the adsorbed protein release by incubating the particles for 10 minutes using a denaturation buffer (50 mM Tris-HCl, pH 8, 8 M urea, 0.1 % Triton-X 100). The resulting samples were analyzed using SDS-PAGE (Figure 4.19).

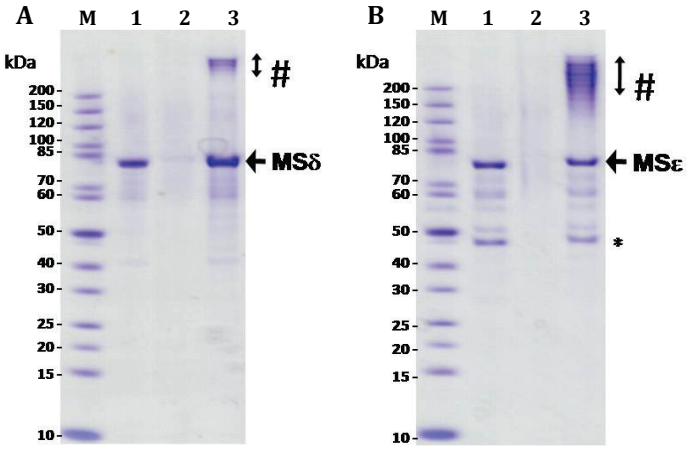

Figure 4.19: Reduced SDS-PAGE analysis of fractions following adsorption and elution of MSδ (A) and MSε (B) using biotin derivatized magnetic supports. *Both gels:* (1) Loaded fraction, (2) wash and (3) elution sample. The arrow indicates the position of monomeric subunits, while # indicates multimeric complexes. * refers to impurities found in the MSε fraction.

As can be seen in Figure 4.19 the biotin derivatized magnetic support had successfully captured both the MSδ and MSε subunits, retained them during the wash, and released them during elution. Since the streptavidin affinity tag must be in a native confirmation for binding, the results indicate that refolding had been successfully achieved using the dialysis approach implemented. A very interesting observation could be made in the lanes containing the eluted samples, as a collection of high molecular weight bands could be identified. In nature streptavidin is found as a tetrameric complex which has the ability to tightly bind four biotin molecules (Sano et al., 1998; Stayton et al., 1999). The results visualized on the SDS-PAGE gel suggest that the covalently attached biotin on the surface of the support may have acted as a scaffold promoting the assembly of a multimeric recombinant complex. In the case of the MSδ, two very distinct high molecular weight bands were seen; while for the MSε a more diffuse range of high molecular weight bands were seen, presumably due to a more disperse nature of the purified subunit. Intriguingly, the high molecular bands have apparently survived the denaturing treatment connected with the elution step (8 M urea/1 mM β-Me) as well as reducing conditions associated with the SDS-PAGE gel electrophoresis, indicating a very strong interaction.

Proteolytic release of assembled F_1-ATPase from the support was part the strategy using biotin-mPVA and to investigate if the AcTEV protease could successfully interact with immobilized MSδ and MSε and mediate the release of δ and ε, a digest was performed. 2 µg/ml biotin-mPVA support was loaded with MSδ or MSε as previously described and subjected to proteolytic treatment using 2.5 u/µg using the buffer recommended by the manufacture (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA) and overnight incubation at room temperature under vigorous shaking.

The SDS-PAGE analysis visualized in Figure 4.20 revealed that the AcTEV treatment had successfully resulted in the formation of new bands, which based on molecular size could be identified as digested fragments originating from MSδ and MSε. However, no subunits or even fragments appeared to have been released from the support as a consequence of the proteolytic activity. In addition to this, a band corresponding to the molecular size of the AcTEV (29 kDa) could also be identified in the fractions, suggesting that the protease had been adsorbed by the biotin-mPVA support.

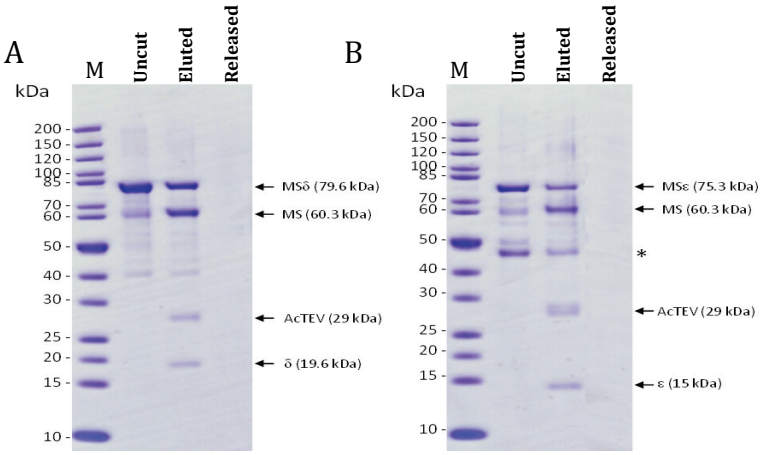

Figure 4.20: Investigation of AcTEV proteolytic digestion of MSδ (A) and MSε (B) subunits immobilized on biotin derivatized magnetic support. Label at the referrers to the following: *Uncut:* Untreated control samples, *Eluted:* Samples eluted from the support using denaturation buffer, *Released:* Sample of the supernatant. The arrow indicates the identity of the bands.

Based on the positive results obtained during the investigation of the adsorption of MSδ and MSε, and the AcTEV digestion of the immobilized MSδ and MSε, the next step in the investigation was to study adsorption of the Hα, Hβ and Hγ/γ by particles preloaded with MSδ or MSε. The aim of the investigation was similar to the previously described study performed with the magnetic amylose beads, namely to verify that adsorption of Hα, Hβ and Hγ/γ were dependent on the presence of the scaffold subunits (i.e. MSδ or MSε) and thus to initially verify correct assembly of the ATPase. Adsorption of the MSδ

and MSε subunits was preformed as described earlier using 20 mg support and after the initial binding and washing steps, the support was divided into 5 mg aliquots, magnetically separated and resuspended in 500 µl of assembly buffer. Control samples containing biotin linked magnetic beads without pre-adsorbed MSδ and MSε were also included in the investigation. To the suspension the various combinations of the remaining denatured subunits were subsequently added in the stochimetric ratio 3:3:1 (Hα, Hβ and Hγ/γ) in a final volume of 8 µl, ensuring at least 60-fold dilution of the 8M urea present. The supports were then incubated at room temperature overnight with mixing by rotation and the following day washed three times before being subjected to 'elution' for 10 minutes using denaturation buffer at room temperature with mixing by rotation. The resultant samples were subsequently analyzed using SDS-PAGE (Figure 4.21).

The SDS-PAGE analysis of the 'eluted' samples from the biotin coupled mPVA supports preloaded with either MSδ or MSε indicated the capture of combinations of Hα, Hβ and Hγ/γ, and also Hα and Hβ in the absence of Hγ/γ , as previously seen. These results were as expected. However in the case of the control, which should not contain any proteins due to the missing linker between the support and the added subunits, bands similar to those seen for supports preloaded with MSδ and MSε were observed. This suggests a high degree of unspecific binding to the biotin coated supports.

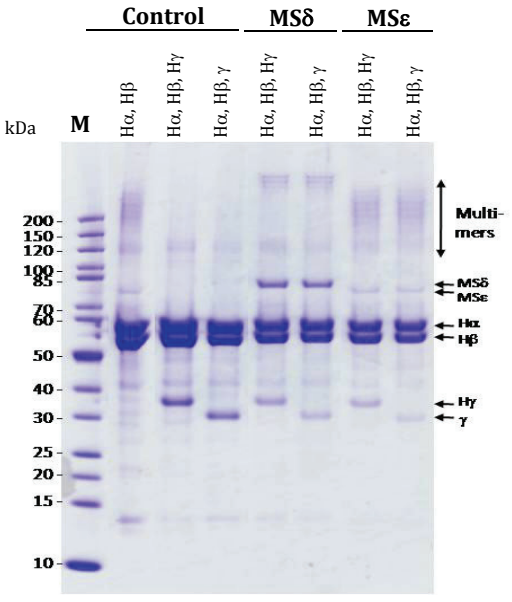

Figure 4.21: Coomassie stained reduced SDS-PAGE analysis of samples showing the assembly of F_1-ATPase subunits using magnetic biotin derivatized magnetic particles. At the top of the gel is noted if the sample arises from supports with an initially bound subunit or a control not preloaded with MSδ or MSε. The combination of subunits included in the second round of adsorption is also shown. The arrows indicate the identity of each band. The vertical arrow indicates the size range of the mulitmeric complexes formed.

The problem of non-specific adsorption could be due to a low biotin ligand density on the prototype supports used, in which PVA base coating or non-coupled sites following activation were present. If so, blocking of the sites of non specific binding may reduce the problem of Hα, Hβ, Hγ and γ subunits binding to the support, rather than assembling to the MSδ or MSε. A method analogous to that used for blocking non specific interactions during western blotting or ELISA assays was used. The experiment above was repeated, however two additional steps of incubation in skimmed milk were added to the process. The procedure of using skimmed milk to block unspecific adsorption was also implemented by Shin et al. (1996) during assembly F_1-ATPase and subsequent capture using GST-tagged δ or ε immobilized using affinity chromatography.

Prior to binding of the MSδ and the MSε subunits, the support was incubated in a MBP buffer solution containing 5% (w/v) skim milk powder (Sigma-Aldrich) for 1 hour with vigorous shaking. The process was succeeded by three repeated wash steps using MBP buffer, followed by a 1 hour incubation period of the blocked supports in dialyzed MSδ and MSε solutions, as previously described.

Unbound protein was removed by repeated washing steps and the support was subsequently incubated once more in a 5% skimmed milk solution. At this point the remaining procedure was conducted as previously described using various denatured combinations of Hα, Hβ and Hγ/γ subunits and the samples were subsequently subjected to three periods of extended washing (3x30 minutes under vigorous shaking) followed by the previously employed method of 'elution' using denaturing buffer.

However, despite the efforts to reduce non specific subunit adsorption using skimmed milk proteins, the SDS-PAGE analysis of the eluted samples shown in Figure 4.22 revealed that the use of blocking had failed to overcome the problem.

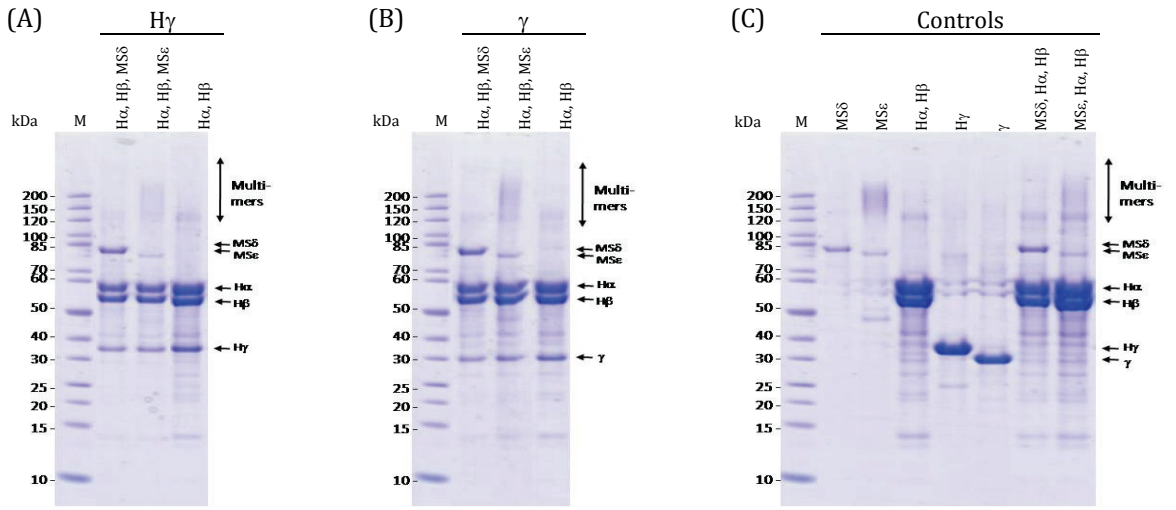

Figure 4.22: Reduced SDS-PAGE analysis of 'elution' samples following assembly using biotin derivatized mPVA supports which had been blocked with skim milk. (A) Assembly performed in the presence of the Hγ-subunit and **(B)** γ-subunit. **(C)** Controls with subunit combinations that should not give assembly: lanes 2, 3, 7 and 8 should only have bands corresponding to MSδ or MSε, all other lanes except lane 1 should have no bands. . (M) Standard protein marker. The arrow indicates the identity of bands. At the top of the gel is noted subunits included in the analysis.

The biotin-mPVA particles which lacked the pre-adsorbed MSδ and MSε (lane 3 in Figure 4.22 A and B) should not be able to absorb the other subunits and thus should have no bands in the elution fractions, however large amounts of proteins were seen. In addition to this, several weak bands of unknown identity could also be seen clearly on the SDS-PAGE gel and it was concluded that these were proteins originating from the skimmed milk solution, further demonstrating the lack of affinity of the adsorbents.

A final attempt to obtain a controlled absorption and to eliminate the nonspecifically adsorbed protein was attempted, in which the washing steps were carried out using both the additional skimmed milk wash and a specialized 20 minute treatment with a MBP buffer supplemented with 0.1% triton-X 100. However, SDS-PAGE analysis (Figure 4.22) showed that use of this nonionic surfactant did not improve the problem of nonspecific binding. Based on all of the results it was concluded that the biotinylated mPVA particles had failed to display the basic requirements of an affinity adsorbent. It was thus decided that the biotinylated mPVA supports available were unsuitable for the

task of serving as a smart handle during protein complex assembly and no further attempts were made.

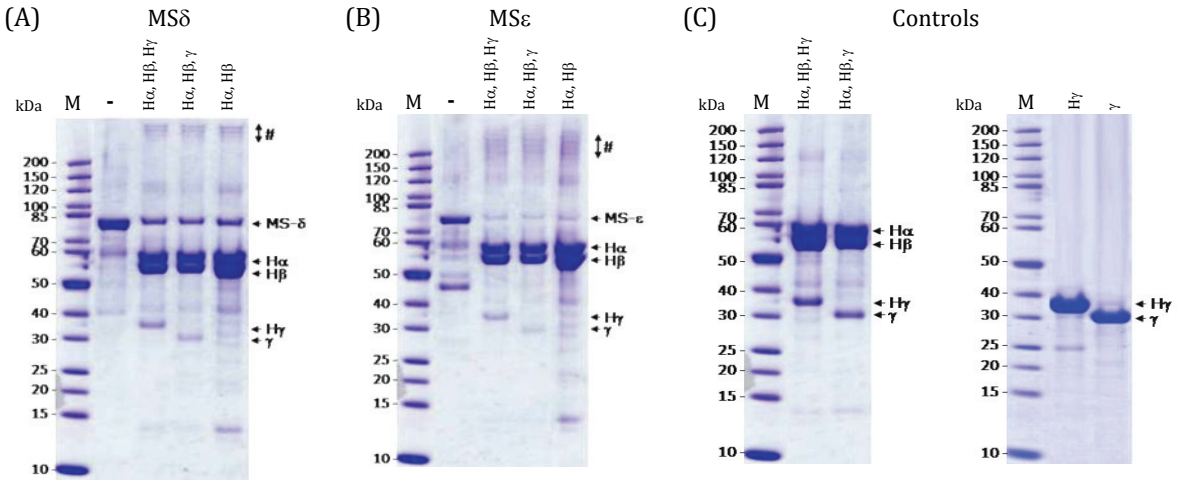

Figure 4.23: Coomassie stained reduced SDS-PAGE analysis of eluted samples originating from the third study of assembly using biotin derivatized mPVA support in which washes with skim milk proteins and triton X-100 were used to reduce non specific binding. (A) Assembly using magnetic support carrying preloaded MSδ, **(B)** support loaded with MSε prior to assembly and **(C)** Controls included no initial adsorption of MSδ or MSε. (M) Standard protein marker. At the top of the gel is noted which subunits were included in the experiment. (-) Indicate that no additional subunits were added after the initial adsorption step. The arrow indicates the identity of each band.

Due to the prototype nature of the biotin derivatised support, no information regarding the performance of it, or the method by which the beads had been derivatized were available and it is not possible to explain why the support had failed. However observations of their behaviour showed the biotinylated mPVA supports did not display any tendencies to form agglomerates in either a protein loaded or unloaded state, which would have indicated a hydrophobic surface. The supports thus appeared to behave in a similar way as other types of mPVA particles used here, including IDA as well as underivatized.

4.5 General discussion

In this chapter recombinant *Escherichia coli* F_1-ATP synthase subunits engineered with an N-terminal HAT tag or N-terminally fused to a maltose binding protein/streptavidin cassette carrying a sandwich His_6 tag were overexpressed and purified using metal-chelate affinity chromatography under denaturing conditions. Dialysis of various mixtures of subunits against an assembly buffer leads to the formation of ATP hydrolyzing activity establishing that correctly assembled F_1-ATPase nano-engines could be formed in free solution. To investigate the possibility of promoting formation of the F_1-ATPase complex using various magnetic supports as smart handles, three strategies were attempted using a step by step approach in which a single subunit was immobilized and used as scaffold during subsequent reconstruction procedure. In the first attempted approach IDA-couple mPGA support were used to immobilize γ through the HAT-tag followed by addition of the remaining subunits and a rapid dilution step converting denative to native conditions. However, as all proteins were found deattached from the support after this step it was speculated that the interactions between HAT-γ and the IDA ligand had been too weak to handle the transition in the assembly buffer and the associated pH value of 6. It was concluded based on the obtained results, that this approach had failed to deliver the intended outcome. The second strategy involved the Maltose Binding Protein tag (MBP) and >10 μm commercial amylose coupled agarose magnetic beads. A similar approach as described previously was attempted using native refolded δ and ε as scaffold and it was found that the subunits could be recovered from the various mixtures in a structurally dependent manner. If MSδ/ε were initially immobilized the α, β and γ subunits could be recovered, while only traces of γ could be seen if no preliminary binding had been performed. However, the binding capacity of the support was found to be very low and despite the use of highly sensitive HPLC analysis, no ATP hydrolyzing activity could be established. Attempts to promote assembly using biotinylated mPVA particles and Streptavidin fused subunits were also conducted however this support appeared to bind indiscriminately and even extensive treatment with skimmed milk and nonionic surfactant proved insufficient to overcome the problem.

However the observation that high molecular weight complexes were formed by MSδ and MSε in the presence of the biotin-mPVA, presumably at the surface of the support, may suggest that these were organized and assembled into specific structures by the support. Formation of complexes was never observed, in free solution and never while using the amylose coupled magnetic beads, which suggests that the biotin-mPVA support may have served as catalyst in the process. Despite the failure of the support as an affinity type adsorbent, the observation that immobilized subunits could, to some extent, be modified by site-specific digestion using the AcTEV enzyme indicated that adsorption had occurred at the surface. The fraction of subunits which remained unmodified could be explained by simple inaccessibility caused by steric hindrance, or by the observation that the AcTEV protease itself may have been absorbed by the support, thus efficiently ending the modification process prematurely.

The properties of the recombinant subunits produced may also have contributed to the less successful results. It is a well known challenge to fuse tags to native proteins and to do this without interfering with the structure and activity of the protein in question. In this study, several reports in the literature were investigated to gain an understanding of the previous successful expression of the F_1-ATPase associated subunits. However, despite this, the expression and the ability to obtain functional complexes still remained a simple question of trial and error as no tool exist which can predict the impact and the outcome. As the choice of tags used in this study was, to some extent governed by the available magnetic supports and ligands coupled to these, further advances in magnetic support technology may lead to better performing adsorbents with a wider selection of coupled ligands in the future.

4.6 Conclusion

The work presented here has provided evidence that the F_1-ATPase from *E.coli* can be assembled on the surface of magnetic supports from the individual recombinant subunits. This evidence was obtained by SDS-PAGE analysis of the fractions from elution of magnetic supports employing maltose binding protein affinity interactions, after assembly had been conducted. Subunits of the ATPase complex were only seen when an initial scaffold subunit (MSδ or MSε) was first adsorbed to the magnetic support surface. However, conclusive proof of the assembly of active F_1-ATPase complexes on magnetic supports could not be demonstrated. In contrast, assembly of active F_1-ATPase complexes from the recombinant subunits used was possible in free solution. It is thus tentatively concluded that active ATPase could be assembled on the magnetic supports, but that the amounts of the complex so assembled were too low for the activity assays employed. Further work should be conducted to confirm and extend these findings.

One of the reasons for the low amounts of active ATPase assembled is concluded to be due to the rapid refolding and assembly strategy employed here. In the current work the complex was assembled from individual denatured subunits, as is also reported by others. However, the approach used for assembly described in the current work differs in one important aspect from all previously described methods for ATPase assembly. In successful previous descriptions (and also the successful reconstruction in free solution in which activity was measured in this chapter) slow refolding/assembly occurred via the gradual replacement of the denaturing buffer through dialysis. Dialysis allows gradual reduction of pH and a slow introduction of important compounds such as ATP and Mg^{2+}, which are known to promote ATPase assembly. In contrast the approach used in connection with assembly on the magnetic beads involved a more rapid and less gentle method by which one buffer was replaced by another through a magnetic separation step and conversion from denatured to native state occurred rapidly. Given that refolding and unwanted aggregate formation in general are described by higher order kinetics, it is believed that the less gentle approach may have substantially reduced yields of active ATPase and that alternative approaches should be devised for future work.

Acknowledgements

Dr. Kim Kusk Mortensen and Dr. Søren Helmark are thanked for supplying plasmids pET-15b-MBP-SA and pSH1, respectively. Dr. Matthias Franzreb is acknowledged for supplying the biotin derivatized mPVA particles and hosting KEO. Dipl.-Ing. Boris Kühl is acknowledged for support with the MALDI-TOF measurements.

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Chapter 5

Conclusion and future work

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5. Conclusion and future work

5.1 Final conclusion

Magnetic adsorbents represent an extremely versatile means of carrying out complex bioprocessing and downstream processing tasks that go well beyond protein purification. They can be used in semi-continuous high-gradient magnetic fishing based processes to stabilize fermentation broths *in situ,* and to act as a support for the efficient manipulation of proteins, which allow controlled chemical modifications to be made as well as protein complexes to be assembled. It has been demonstrated that many steps using magnetic supports can be conducted continuously, and it is expected that when new types of HGMF separators become commercially available (currently in development elsewhere), truly continuous processes will be a reality. A common issue that needs to be addressed for any process using magnetic particles is the suitability of the base magnetic support itself for the process (e.g. sterilisability, corrosion compatibility with buffers and reagents, reactivity with chemicals) as well as the means of attaching the proteins to them. Suitable types of base particle can most likely be developed with relative ease and can be expected to be rather generic, however it is expected that the protein attachment mechanism may prove more of a challenge, being much more process specific.

HGMF processing can be used to selectively remove trace amounts of troublesome proteases directly from a fermenter during a cultivation. This can be done with minimal effect on the fermentation itself and no obvious effects on the bacterial cells. The stability of protein product of interest can be dramatically enhanced by the process. Whilst a spiked model protein was used in the current studies, the principles demonstrated here are adaptable to fermentations with other organisms and products of interest. Further work should be aimed at demonstrating the performance of the approach in a genuine fermentation process experiencing real product stability problems. Given that low amounts of adsorbent are required, this could be demonstrated at pilot scale with little difficulty.

Controlled semi-continuous PEGylation of proteins is possible using magnetic supports as a handle to manipulate the protein. Whilst the concept was successfully

demonstrated for the PEGylation of trypsin, it is expected that it is widely adaptable to the modification of other proteins with other types of chemicals, polymers and substrates. A very small scale process was demonstrated in the current work, in which staggered herring bone mixing systems were used and flow rates were in the microliter to milliliter range. This shows the applicability of magnetic supports and HGMF to micro-scale and 'lab on a chip' type applications, however, on the other hand it should be realized that all of the components of the process demonstrated here are scalable. For example continuous pipe reactors are a staple of the process engineer's toolbox and can be obtained in any size needed.

The use of a magnetic support based strategy for *in vitro* assembly of a biological complex was demonstrated. Evidence for the assembly of the F_1-ATPase on magnetic supports from individual subunits was found, although activity could not be conclusively shown. It is nevertheless concluded that the overall concept of an assembly line for biological complexes, nano-engines etc based on magnetic supports has merit, and that further work should be conducted to conclusively prove the principle. Given the work presented here, the F1-ATPase model is considered to be suitable for such a demonstration, although the strategy used should be adjusted.

5.2 Future work

5.2.1 Avoiding proteolysis in fermentation broths by using HGMF

The demonstration of HGMF as a powerful *in situ* tool for direct removal of proteases during a fermentation process was very successfully demonstrated in chapter 2 using *Bacillus* cultures and spiked BSA. The next natural step in the development of the application would be to demonstrate the stabilization of a recombinant heterologously expressed protein and thus to show a more realistic system. One example could be the expression of a pharmaceutically interesting peptide like insulin, which is traditionally produced using genetically modified strains of *Saccharomyces cerevisiae* or *Escherichia coli*. It is highly likely that a process involving expression of insulin and subsequent secretion to the medium using an unmodified *Bacillus* strain would result in degradation and thus seriously impact the yield and the quality of the product. Through a demonstration similar to the one presented in chapter 4, it would be possible to investigate the impact on the stability of insulin and thus to present data obtained from more realistic conditions.

From an engineering process perspective, the use of different adsorbent addition and collection strategies should be examined. This should encompass a study of how the number of adsorbent addition and recoveries made can be used to further extend the stabilization of the protein. Additionally, the recovery of the adsorbent directly from the fermenter via a recirculation loop, which fed the broth directly back to the fermenter should be tested (figure 5.1), rather than the approach used in the current work, in which the broth was collected in an intermediate vessel. New magnetic separators such as the centrifuge-based HGMS system (Stolarski et al., 2008) briefly described earlier, should be used to allow a more rapid collection process compared to the one used in chapter 2 (figure 5.1).

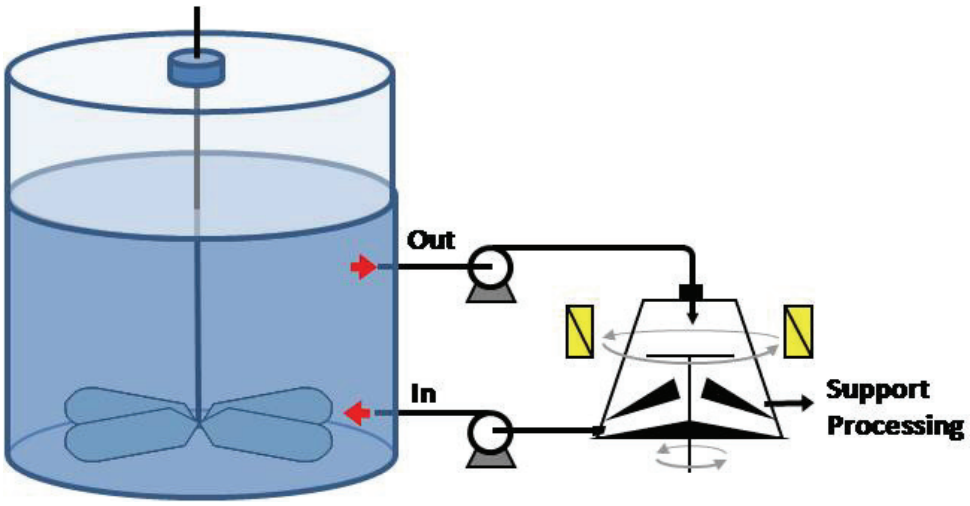

Figure 5.1: Schematic of a fermentor equipped with a recirculation loop and centrifuge-based continuous HGMS system.

A major stumbling block to implementation of the protease removal process at pilot or large scale is obtaining an appropriate adsorbent, which is highly selective for the proteases causing the problems. In the current work, a broad spectrum serine protease binding ligand was used, and this should be considered as the first choice for developing a process for a genuine fermentation problem. However, it is realised that ligand screening and perhaps ligand development may be required for other fermentation systems, which secrete different types of protease. An additional consideration before implementation in pilot and large scale processes is the ability to adequately sterilise the adsorbents. In the current work, ethanol soaking proved suitable, however more rigorous methods are likely to be required in an industrial setting. This is a significant challenge, since the base adsorbent itself must tolerate the sterilisation process, as must the ligand. Development of heat tolerant adsorbents would permit conventional steam sterilisation, and should be considered a priority. However, chemical means may prove to be a suitable interim solution. Given that the manufacturing process itself most likely kills any microorganisms present, aseptic adsorbent collection after manufacture may be suitable for a single use adsorbent.

5.2.2 Continuous PEGylation

One of the major challenges connected with setting up the semi-continuous process presented in chapter 3 was associated with the scale of the system and the need to obtain sufficient mixing, rather than the application itself. The mixing challenge was especially problematic during the PEGylation process due to differences in viscosity of the liquids; however the problem could be overcome through the use of staggered herringbone mixers. As it is highly unlikely a similar problem would be faced in a scale-up situation, the presented results clearly open up the possibility of studying PEGylation in pilot scales. In addition to this, novel HGMS technology like continuous selective High Gradient Magnetic Separations based on the use of centrifugation (Stolarski et al., 2008) could serve to develop the process into a truly continuous operated system. A possible extension of the process with a system like the centrifuge-based HGMS is shown in figure 5.2

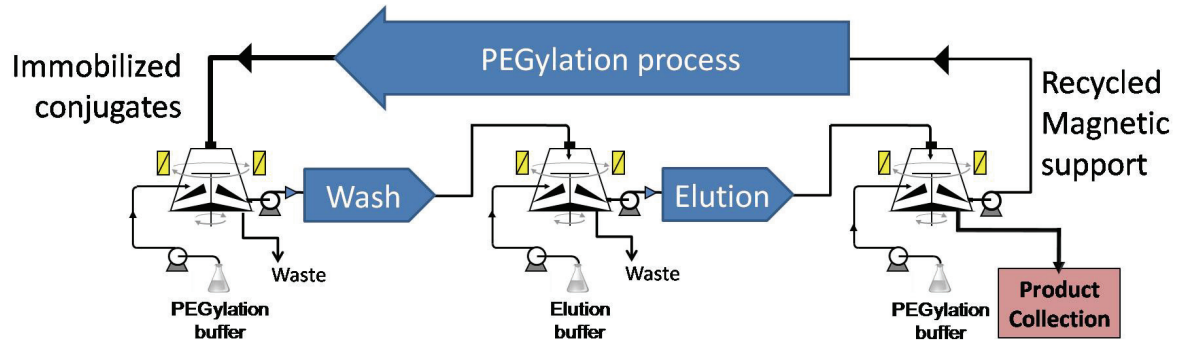

Figure 5.2: True continuous magnetic separation using a rotating matrix centrifuge. The figure illustrates a process for the separation and elution steps as a continuous process based on the implementation of rotational matrix centrifuge based-HGMS technology (see Stolarski et al., 2008). A possible implementation could be envisioned in the following way: After the PEGylation step the magnetic support would proceed to the first separation step, during which the supernatant resulting from the PEGylation step would be replaced with PEG buffer. The next process would be washing step aimed at the removal of any trace amount of PEG, followed by a new separation procedure. The separated support would this time be resuspended in elution buffer ensuring release of the formed conjugates during the subsequent retention period. Finally during the last process step the magnetic support would be separated from the eluted conjugates and the particles now resuspended in PEG buffer circulated back into the system ready to be processed again.

5.2.3 Assembly of a nano-machine

Formation of a protein complex from denatured subunits to a fully assembled structure is a process which may be described as series of parallel events leading to the native state of the individual subunits and the complex itself (Dill and Chan, 1997). The subunits used in the current work were expressed as insoluble inclusion bodies, which needed to be solubilised and were thus present in an unfolded and denatured state. One of the major differences between previous successful assembly studies and the approach attempted here is the speed by which the conditions are changed from denaturing to native. The traditional approach has been through the use of dialysis which ensures a slow exchange of the buffers and thus a slow conversion to the native conditions. The rapid nature of the approach implemented here may have contributed to the difficulty in measuring activity of the complex by promoting misfolding of the subunits and thus failure to achieve formation of active F_1-ATPase. The rapid dilution procedure however, is much more straightforward when using a HGMF-based approach as compared to a slower rate. Nevertheless, in future studies the issue of dilution and especially the rate of change of conditions should be examined.

The use of the support types chosen in this study also needs to be re-evaluated. The decision to use the three types of support included in the study, and thus the affinity tags chosen, were to some extent governed by which magnetic supports were available. However, it does seem clear that the use of a full sized streptavidin tag and biotin ligands was not suitable due to the extreme affinity and tendency to form multimeric complexes. The traditional use of the streptavidin/biotin systems is somewhat different to that used in this study, as the protein is usually immobilized on the support while the target molecules are biotinylated. However, due to the random nature of biotinylation caused by linking chemistry which results in positional isomers on a protein, and thus the lack of control over subunit orientation, the approach was abandoned. Instead future studies should consider using the peptide based Strep-tag II, which would allow streptavidin to be immobilized on the support instead of being expressed as part of the protein. This would eliminate the need for proteolytic release of the complex from the support, as well as problems like undesirable formation of multimers and solubilization issues experienced in this study (Terpe, 2003). Assembly based on the use recombinant

subunits carrying only the MBP tag, or alternatively the chitin binding domain, should also be investigated using either amylose coupled or chitin linked beads, respectively.

5.3 References

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Chapter 6

Appendices

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6. Appendices

The appendix section contains supplementary materials connected to the research chapters (chapter 3 and 4). Appendix A describes schematically how the batch part of the semi-continuous PEGylation system, which included separation, wash and elution steps were operated.

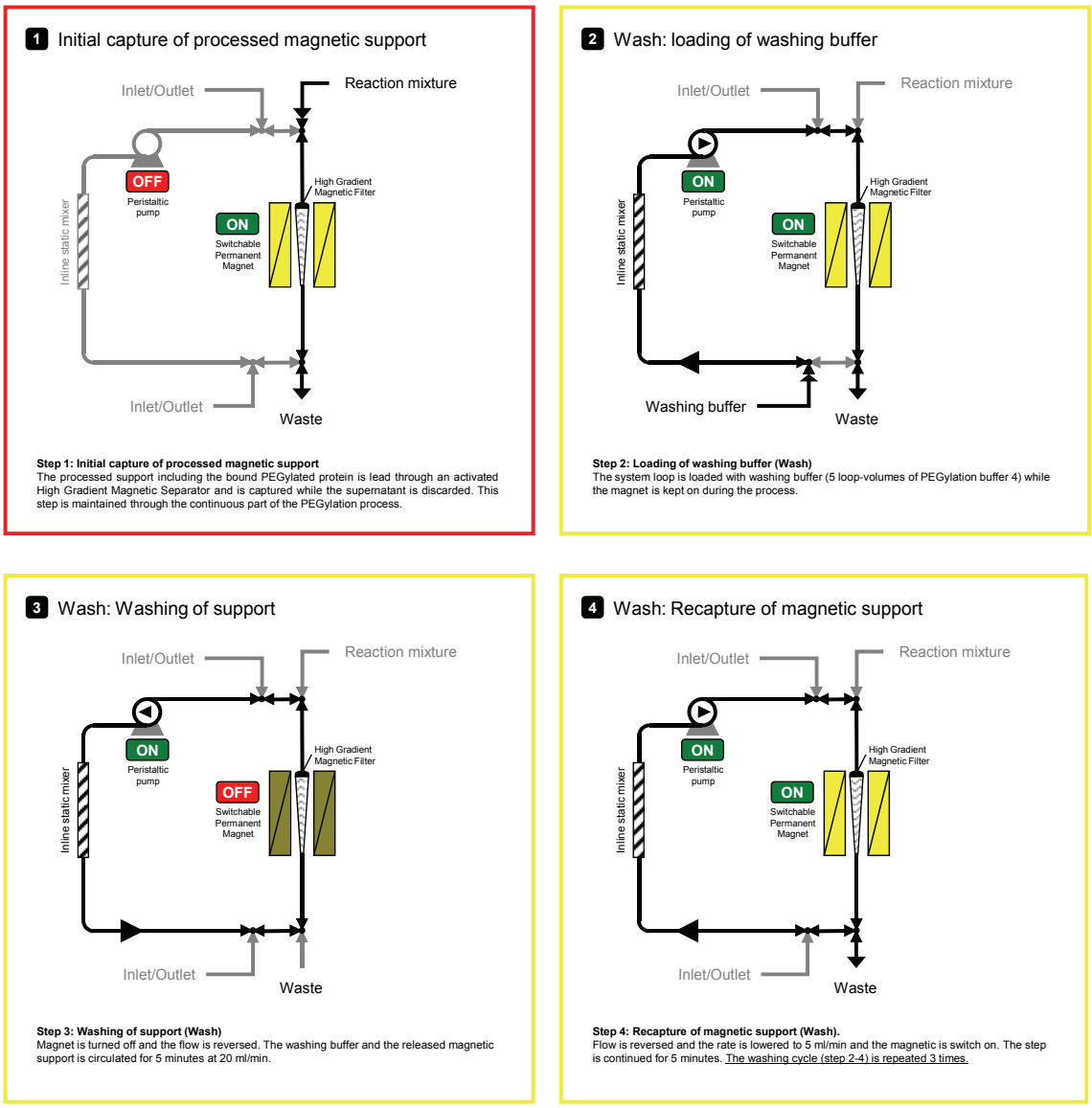
Appendix B introduces two earlier PEGylation systems on which the system presented in chapter 3 is based. The systems are described and studied and a short conclusion is made on the basis of the collected results.

The contents of appendix C are connected to chapter 4 and contain additional materials associated with the preparation of the vector constructs, which served as carriers of the DNA encoding the manipulated F_1-ATPase genes. This material includes analysis of the plasmids using endonucleases (Restriction Fragment Length Polymorphism), restriction maps and more detailed information on the purification process of the F_1- ATPase subunits, including chromatograms and SDS-PAGE analysis.

The last appendix, denoted D, includes a peer-reviewed paper, which describes a novel approach to *in situ* removal of a peptide product based on the use of magnetic supports and the concept of High Gradient Magnetic Fishing. The research included in the paper was primarily carried out by Tobias Käppler and Martin Cerff under the supervision of Professor Clemens Posten at the University of Karlsruhe, Germany, however large scale preparation of the magnetic supports was carried out as an aside during the work conducted in the Ph.D. presented here.

6.1 Appendix A: Operation of the batch mode steps of the HGMF PEGylation system

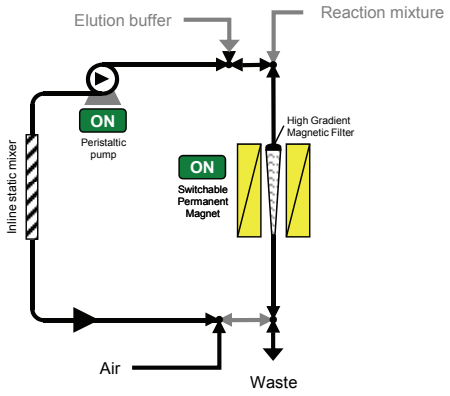
Schematic figures showing each of the batch mode steps included in the HGMF-based semi-continuous PEGylation process are shown below. Black lines indicate the active part of the process, whilst light gray parts indicate that part of the process is not in use. For details see figure 3.4a.



Border colour legends

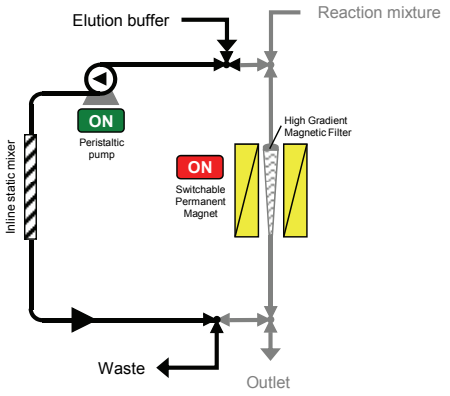
Initial capture of the process support	Elution steps
Washing steps	System regeneration

5 Wash: Final removal of washing buffer (PEG4 buffer)



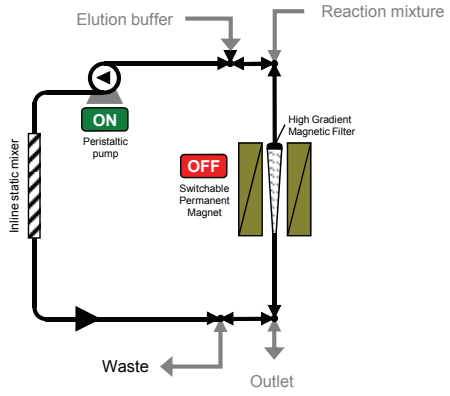
Step 5: Final removal of washing buffer (PEG 4 buffer)
The washing buffer is forced out by slowly pumping in air into the system.

6 Elution: Loading of Elution buffer



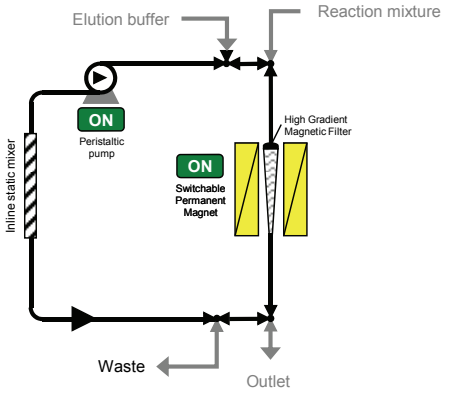
Step 6: Loading of Elution buffer
Alternative loading path is used and the system is loaded with 10 ml elution buffer.

7 Elution: Elution of PEGylated Conjugates



Step 7: Elution of PEGylated conjugates.
The magnet is switched off and the pump set at maximum speed (20 ml/min) and the liquid is pumped around the system for 5 minutes ensuring elution of the reversibly immobilized conjugates.

8 Elution: Particle recapture

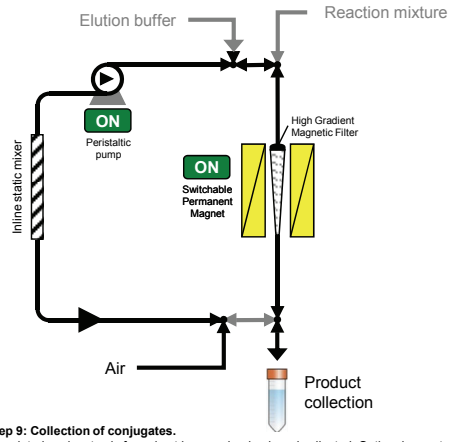


Step 8: Support is recaptured
The magnetic is switch on, the flow reversed at 2 ml/min for approximately 5 minutes and the magnetic support is recaptured in the magnetic filter.

Border colour legends

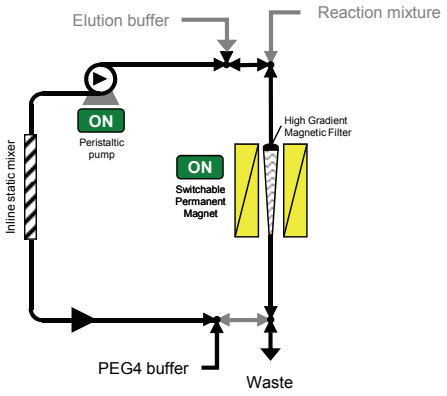
Initial capture of the process support	Elution steps
Washing steps	System regeneration

9 Elution of PEGylated Conjugates



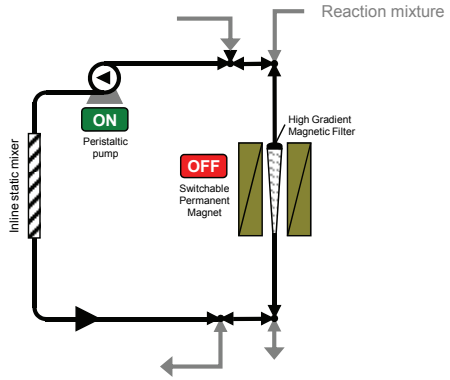
Step 9: Collection of conjugates.
The eluted conjugates is forced out by pumping in air and collected. Optional repeat of the elution step (step 6-9).

10 System regeneration: Loading of PEG4 buffer



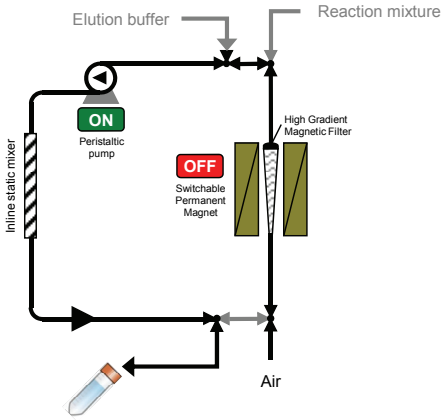
Step 10: System regeneration - Loading of PEG4 buffer
The system if flooded with PEG4 buffer for 5 minutes at 20 ml/min

11 System regeneration: Particle release



Step 11: Release of the magnetic support
The magnet is switched off, the pump flow reversed and set at maximum speed (20 ml/min). The liquid is pumped around the system for 5 minutes to ensure particle release.

12 System regeneration: Particle recovery



Step 12: Recovery of the magnetic support.
The pump is decreased to 2 ml/min and the released magnetic support is forced out of the system using air. The regeneration step was repeated three time (step 10-12) to ensure efficient particle recovery.

Border colour legends

Initial capture of the process support	Elution steps
Washing steps	System regeneration

6.2 Appendix B: 1st and 2nd generation semi-continuous PEGylation systems

Prior to the assembly of the HGMF-based semi-continuous PEGylation system presented in chapter 3, two prototype systems, denoted generation one and two, were designed on the basis of the experimental evidence collected at the time. The investigation of the performance of the two earlier systems was primarily aimed at the study of adsorption and PEGylation using various means of mixing.

1st generation HGMF-based semi-continuous PEGylation system

The first generation HGMF-based semi-continuous system was built on the concept of the kinked pipe reactor design described by Ferré (2005), which is based on the assumption that a series of 90 degree kinks may serve to achieve sufficient turbulence and thus mixing, under conditions which otherwise favour a laminar flow pattern. The kinked pipe reactor employed in this study was prepared using 1 meter of Teflon tubing with a 1 mm diameter attached to a wooden support and included 45 ninety degree turns corresponding to one positioned at every 22 mm. To promote delivery of the reagents two 1 ml samples loops were connected to the kinked pipe reactor via motor valves (MV-7, Pharmacia). To ensure a smooth flow through the system, two reciprocal pump systems (P-500, Pharmacia, discontinued GE Healthcare, Chalfont st., Giles, UK) were also connected to the motor valves and served to drive the reagents out of the sample loop and into the system. The two pumps were each set at 0.5 ml/min resulting in a combined flow rate of 1 ml/min through the system. To extend the retention time, additional Teflon tubing was attached to the kinked pipe reactor and during this part of the process no active mixing was included. The length of the Teflon tubing used during the adsorption process was 5.5 meters, while 9 meters was employed during the PEGylation step, resulting in residence times of 5.1 and 7.9 minutes, respectively. To monitor the progress of the magnetic support travelling through the system, UV detectors were placed before the kinked pipe reactor and at the process exit point. A schematic overview of the first generation HGMF-based semi-continuous PEGylation system is available in figure B1.1. All reagents, including the system buffers were based on PEGylation buffer 2 (133 mM Sodium borax, pH 8, 10 mM $CaCl_2$).

Abbreviation	
MP	Magnetic particles
MP-T	Trypsin preloaded magnetic particles
MV#	Motor value
P4B	PEG4 buffer
RP#	Reciprocal pump
RT	Retention tube
SC-mPEG	SC-mPEG solution
T	Trypsin
UV#	UV detector

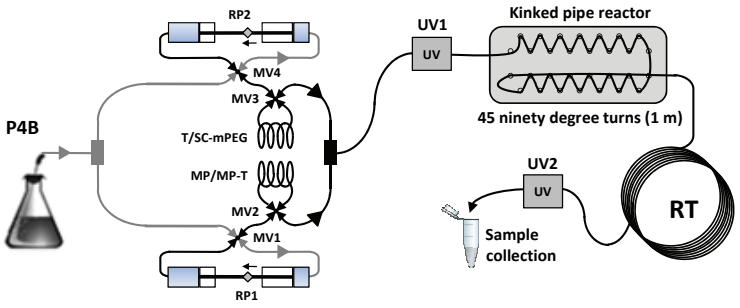


Figure B1.1: Schematic presentation of the first generation PEGylation system. The system was built around the use of a kinked pipe reactor design based on Ferré (2005). During analysis of the adsorption step, the length of the retention tube was 5.5 meters, while a 9 meter tube was used during investigations of the PEGylation step. Pump speed was set at 2x 0.5 ml/min resulting in process times of 5.1 and 7.9 during adsorption and PEGylation respectively. To determine the exact process times UV detector units were employed during the set of the system, however only the exit unit (UV2) remained on during sampling.

To study the adsorption step using the first generation system, the two samples loops were filled with either 4 mg/ml magnetic support or a trypsin solution with a concentration ranging from 0 to 4 mg/ml. All concentrations were diluted 2-fold as a consequence of the flow merger and through the readings obtained by the UV units, the appropriate timing of sampling was determined[6]. Collection of the processed magnetic support was performed using a microtube placed in a magnetic rack and after 30 seconds sampling, the collected product was immediately spun using a centrifuge for 30 seconds at 13000 rpm, and a pipette used to secure a sample. The protein concentration was subsequently determined using the BCA method as described under the methods and material section in chapter 3.

[6] The sampling was initiated 30 seconds after max reading was observed and continued for 30 seconds.

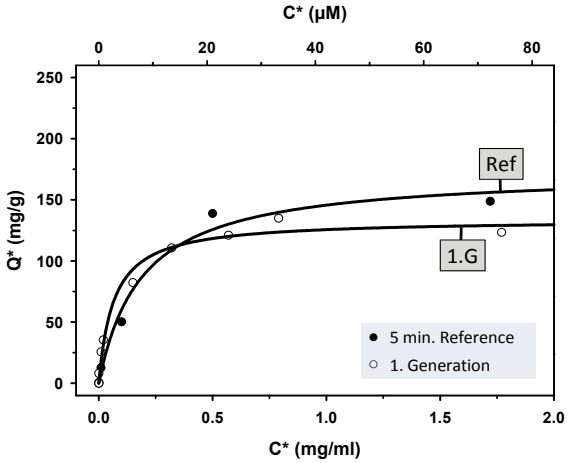


Figure B1.2: Adsorption isotherm obtained for the first generation PEGylation system. The protein concentrations were collected using the BCA method (see materials and method chapter 2) and the data points were fitted to the Langmuir equation. Below is listed the derived Langmuir parameters including a 5 minute reference data set.

	Q_{Max}		k_d				Q_{Max}/k_d
	mg/g	Std_{Err}	mg/ml	Std_{Err}	µM	Std_{Err}	l/g
1. gen	133,9	5,78	0,066	0,016	2,8	0,7	2,0
5. ref	173,2	12,5	0,189	0,052	7,9	2,2	0,92
Determined with BCA							

As can seen in figure B1.2 the performance of the adsorption step using the kinked pipe reactor system delivered a result, which gave a lower maximum binding capacity (Q_{max}) compared to the reference. However the dissociation constant (k_d) was much lower than the reference, and thus an increased tightness of binding (Q_{max}/k_d) was observed using this adsorption system. Based on these results the kinked pipe reactor design appeared to work as expected and even though a decreased binding capacity was observed, mixing was achieved despite unfavourable flow conditions[7].

To investigate the performance of the kinked pipe reactor during the PEGylation step the system was reconfigured with longer Teflon tubing to ensure an increased residence time. During the study, 2 mg/ml preloaded magnetic supports (high load) and SC-mPEG solutions at various concentrations were employed, and the process was preformed as described above fort the study of adsorption. 1 ml of the processed conjugates was collected in a 15 ml falcon tube, which prior to the sampling, was filled with 10 ml PEG2 buffer, serving to dilute the reaction. The samples were subsequently placed along a bar magnetic and the magnetic support was separated from the supernatant. The isolated support was then washed and eluted as described in chapter 2 and analyzed using SDS-PAGE.

[7] The Reynolds number calculated for a flow rate of 1 ml/min in a tube with a 1 mm diameter under the assumption that the liquid has the same physicochemical properties as water is 3.5, and this value equals the characteristics of laminar flow.

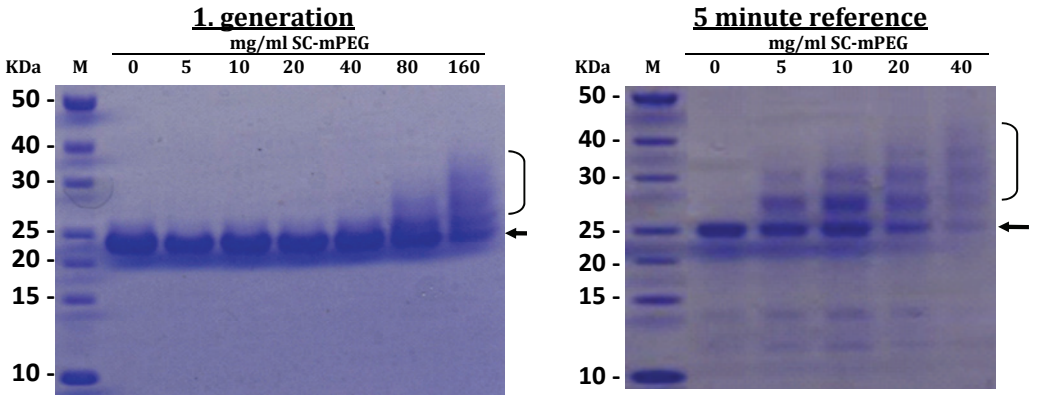


Figure B1.3: SDS-PAGE analysis of PEG conjugates prepared. *Left:* PEGylated trypsin conjugates prepared using the kinked pipe reactor system and a retention time of 7.9 minutes. *Right:* 5 minute reference results obtained using reactions in well shaken microtubes. At the top of the gels are listed the actual concentration of SC-mPEG employed during the experiment. The arrow indicates the band corresponding to native trypsin, while the brackets indicate where PEG conjugates are be observed.

The results of the SDS-PAGE analysis revealed that the first generation HGMF-based system had failed to deliver the expected results. As compared to the reference microtube based, 20 times higher concentrations of SC-mPEG were needed to achieve a comparable PEGylation profile when the reaction was performed using the kinked pipe reactor based system. This result was far from that expected and the most likely explanation for the poor performance of the PEGylation step is that the kinked pipe reactor is unsuitable. The mixing action appeared to be adequate when dealing with flows of equal viscosity during adsorption, but was inadequate for the PEGylation step under which the two streams of reagents had different viscosities. Based on the results obtained for the PEGylation step using the kinked pipe reactor design, it was clear that better mixing was needed to perform this step efficiently. In addition to the short comings of the kinked pipe reactor system, the general approach using reagents contained in sample loops was also found to be problematic. As the reagents are driven into the system by the buffer travelling behind the mixture, a dilution occurs. This notion was supported by readings from the online UV detector, which revealed that particles could be detected passing the exit by a factor of 1.45 longer than expected.

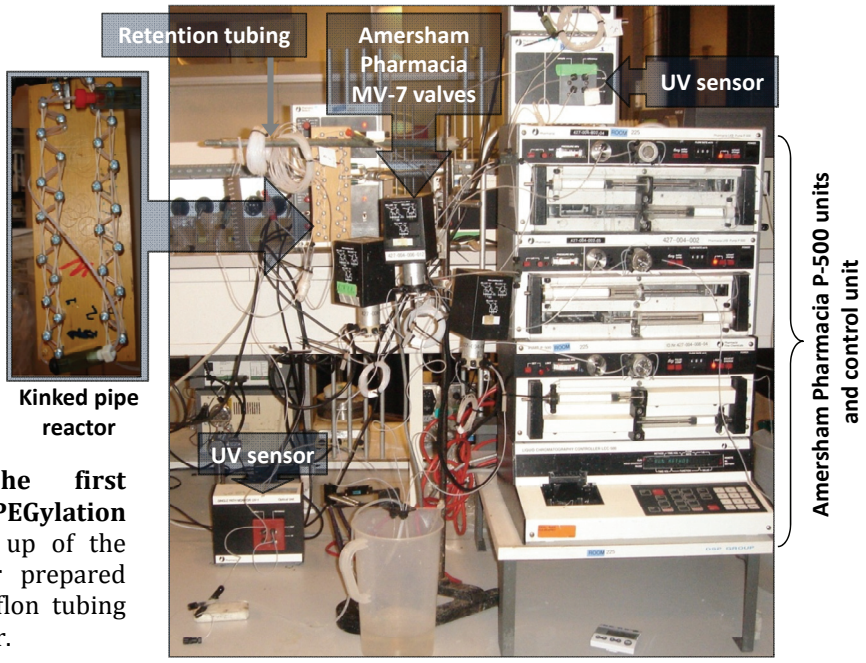


Figure B1.4: The first generation PEGylation system. Left: Close up of the kinked pipe reactor prepared from 1 meter of Teflon tubing with a 1 mm diameter.

2nd generation HGMF-based semi-continuous PEGylation system

Based on the results obtained for the first generation HGMF based semi-continuous PEGylation system, it was clear that a better performing mixing system was needed to enhance the reactions. To achieve this goal, a second generation setup was designed around the use of silicon chip based mixer units (SCM), prepared and designed by Torsten Lund-Olesen (Department of Micro and Nanotechnology, Technical University of Denmark). The SCM units, originally used as part of a lab-on-chip related study, incorporated staggered herringbone structures aimed at achieving turbulent flow (mixing) at low flow rates. Further reading on the design and testing may be found in Lund-Olesen (2008). The second generation HGMF-based PEGylation system was also simplified as compared to the first generation. The previously employed Pharmacia motor valves, reciprocal pumps and sample loops were replaced by a dual syringe pump based supply system (Harvard Syringe pump 22, Harvard Apparatus, 84 October Hill Road, Holliston, Massachusetts 01746, USA). To ensure a uniform distribution of the magnetic support contained in the syringe and thus an even supply, glass beads were added and the dual syringe pump was placed on a shaking table. To counter clogging

problems observed in connection with use of the SCM units, an ultrasonic bath was including in the system to ensure a smooth running process.

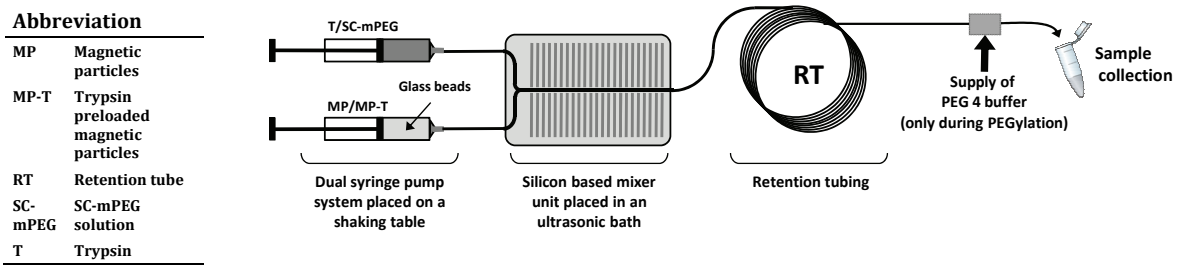
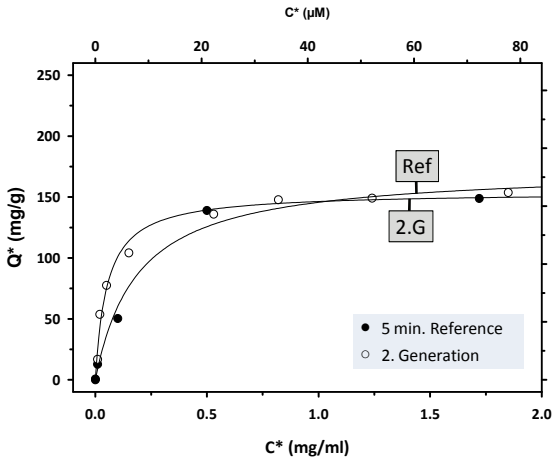


Figure B2.1: Second generation PEGylation system. The system was built around the use of silicon chip based mixing units and the supply of reagents was achieved using a dual syringe pump. To ensure even distribution of the magnetic supports in the supply syringes, glass beads were included in the syringe and the setup was placed on a shaking table. In addition to this, an ultrasonic bath was employed to avoid clogging of the chip based mixing system.

As previously described, the adsorption and PEGylation steps were investigated individually, and in both cases the system was allowed to reach equilibrium by allowing a full system volume to be processed prior to any collection of samples. During the study of the second generation setup PEG4 buffer (100 mM Sodium borax, pH 8, 10 mM $CaCl_2$) was extensively used. Adsorption of trypsin onto the magnetic support was investigation using 4 mg/ml magnetic support and various concentrations of trypsin, and the pumping speed was adjusted to 113 µl/min resulting in a combined flow of 226 µl/min. To achieve sufficient residence time, the SCM unit was attached to 150 cm of Teflon tubing with a 1 mm diameter giving rise to a process time of 5.2 minutes. Sample collection was continued for 5 minutes at the exit by directing the liquid down the side of a 15 ml tube attached to a bar magnetic, resulting in a rapid separation of the magnetic support and the supernatant (the latter was collected at the bottom of the tube). The samples were subsequently analysed using the BCA method as described in chapter 3 and used to prepare an adsorption isotherm.

Figure B2.2: Adsorption isotherm obtained for the second generation PEGylation system. The protein concentrations were determined using the BCA method (see materials and method chapter 2) and the data points were fitted to the Langmuir equation. Below is listed the derived Langmuir parameters, including a data set for a reference employing microtubes well shaken for 5 minutes.

	Q_{Max}	k_d		Q_{Max}/k_d
	mg/g (Std_{Err})	mg/ml (Std_{Err})	µM (Std_{Err})	l/g
2. gen	**154.1 (4.46)**	**0.053 (0.008)**	**2.2 (0.3)**	**2.9**
5. ref	**173.2 (12.5)**	**0.189 (0.052)**	**7.9 (2.2)**	**0.92**



As can be seen in figure B2.2 the second generation mixing system performed better as compared to the first generation setup (see figure B1.2) and the maximal binding capacity was found to be 154.1 mg/g in contrast to 134 mg/ml for the first generation,. The second generation system had a capacity equivalent to 90% of the value observed for the reference system in microtubes well shaken for 5 minutes. In addition to , the dissociation constant (k_d) was found to be similar to the value obtained for the first generation setup. Furthermore the tightness of binding was found to be almost 3 times greater than the corresponding reference sample and approximately 50% higher than for the first generation system. These results all appeared to indicate that the SCM units had performed the task of mixing better, as compared to the kinked pipe reactor design employed in the first generation setup. However, the investigation of the performance had also revealed that operations involving magnetic support concentrations of 2 mg/ml (2-fold dilution of the original 4 mg/ml) gave rise to clogging problems, which were not completely solved by the action of the ultrasonic bath.

To explore the PEGylation step using the second generation setup and the SCM mixing unit, the system was reconfigured to fit the needed requirements. The syringe pump was readjusted to 226 µl/min resulting in a combined flow rate of 452 µl/min, and to ensure a residence time of approximately 5 minutes, 300 cm of Teflon tubing was attached to the SCM unit. In addition to this 5 ml/min buffer was supplied by a reciprocal pump system (P-500, Pharmacia,) at the exit point of the retention tubing. This step was included to ensure a rapid 10-fold dilution of the mixture serving to slow down the PEGylation reaction during the separation step. The PEGylation process was

investigated using various concentrations of SC-mPEG and trypsin preloaded magnetic supports supplied at 2 mg/ml (diluted 2-fold during the PEGylation reaction). After the initial separation of the magnetic support, which was performed using a bar magnet, the conjugates were processed as described under materials and methods in chapter 3 and analyzed using reduced SDS-PAGE analysis.

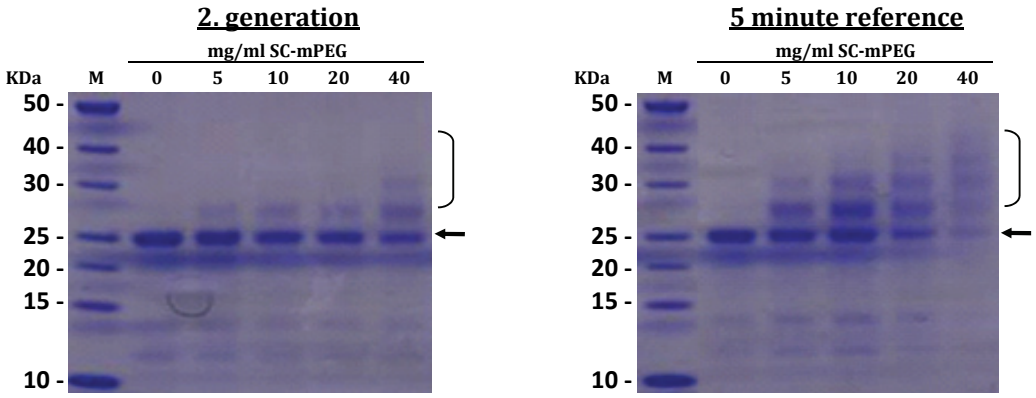


Figure B2.3: SDS-PAGE analysis of the conjugates obtained using the second generation PEGylation system. *Left:* PEGylated trypsin conjugates prepared using the SCM unit system and a residence time of 5 minutes. *Right:* 5 minute reference results obtained using microtube based approach. At the top of gels are listed the actual concentration of SC-mPEG employed during the experiment. The arrow indicates the band corresponding to native trypsin, while the brackets indicate were PEG conjugates may be observed.

As can clearly be seen in figure B2.3 the SCM based system performed far better than the first generation system (see figure B1.3) and at concentration of 5 mg/ml, conjugates can be identified on the SDS-PAGE, however an increased concentration of SC-mPEG did not appear to result in the formation of additional conjugates. Only at a concentration of 40 mg/ml SC-PEG were two bands seen, relating to PEGylated trypsin and this result should be compared to the reference gel where as many as 4 four bands corresponding to conjugates may be identified. Even though the results obtained for the second generation process design were clearly an improvement with regards to performance, the system still remained inferior as compared to reference results. As it was clear that the Staggered Herringbone structures included in the SCM units could achieve the flow mixing needed, the most likely reasons why the system failed to deliver better results were connected to the fact that mixing was limited to the initial parts of the process (i.e. only in the mixers and not in the tubes included to extend the residence time). In addition to this, it was also found that the SCM units lack the capacity to deal with the

concentrations of magnetic support employed, and even though the ultrasonic bath clearly help to solve the problem, a smooth running process was never really obtained. However, despite the evident problems connected with the earlier developed systems large amounts of data were collected and the experience gained from the experiments was used to setup and design the SHM units used in the process described in chapter 3.

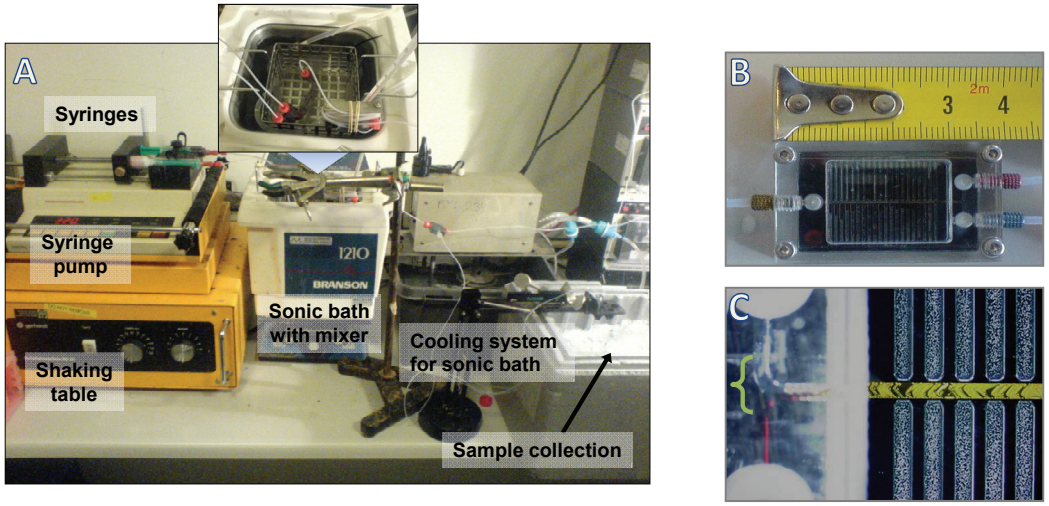


Figure B2.4: The second generation HGMF-based PEGylation system. (A) An overview of the laboratory setup. **(B)** The silicon chip based mixer equipped with two inlets and a single outlet. **(C)** Close up of the staggered herringbone mixing structures. The picture shows flow testing of a pH-indictor being mixing a flow of acid. The bracket indicates the point of merger between the two flows.

References

1. **Lund-Olesen T, (2008)**
On-Chip Biological Analysis using Magnetic Beads
Ph.D. thesis 2008, Technical University of Denmark, Lyngby, Denmark.

2. **Ferré, H, (2005)**
Development of Novel Processes for Protein Refolding and Primary Recovery.
Ph.D. thesis 2005, Technical University of Denmark, Lyngby, Denmark

6.3 Appendix C: Supplementary material for Chapter 4

This appendix contains auxiliary materials connected to chapter 3. When preparing a strategy aimed at proving an easy one step purification of proteins, the best choice in most cases is to employ affinity chromatography. If no natural receptor or binding site is present in the protein in question it becomes necessary to introduce an affinity tag through DNA manipulation. The right choice of tag and the location within the primary sequence may subsequently affect activity of the produced recombinant protein due to negative effects on folding, stability, solubility, interactions with substrates and other proteins, as well as on the expression, both at the transcriptional and translational level. However, as the effects on the integrity of a given target protein, caused by changes to the primary sequence, may be difficult to predict getting it right is often a question of trial and error.

Several naturally existing, as well as modified commercial affinity purification systems exist, and one of the most successful is the polyhistidine tag, which combined with Immobilized Metal Affinity Chromatography (IMAC; Porath et al., 1975), has proven itself in several reports as a simple, fast and highly robust method. The recombinant F_1-ATPase subunits used in chapter 3 were all purified using the IMAC approach and target proteins were either equipped with an amino-terminal fused Histidine Affinity Tag (HAT) in the case of the alpha, beta or gamma subunits, or in the case of a the delta and the epsilon subunits with a small sandwich polyhistidine tag (his_6).

Tagging of the alpha, beta and gamma subunits

Several reports in the literature describe successful expression and subsequent assembly of functional ATP hydrolysing activity prepared from subunits carrying either N- or C-terminal tags. In case of the α and γ subunits a HIS_{10} polyhistidine tag was successfully implemented by Steinemann et al. (1995) at the N-terminal, while Johnson et al. (2006) demonstrated fusion of a similar tag in the N-terminal of the β-subunit. In addition to this, the intriguing study by Noji et al. (1997) in which a direct observation of the rotation of the F_1-ATPase was reported, also used N-terminal polyhistidine tagged β-subunits to achieve immobilization on a glass surface. Successful polyhistidine tagging of

the C-terminal has also been reported in the case of both the β- and γ-subunit (Senior et al., 2006, Ekuni et al., 1998) and it has also been shown that the γ-subunit could be fused to a 27 kDa GFP domain at the C-terminal and still assemble into an active complexes (Prescott et al., 2003). However, tagged γ-subunits have also been reported to lose activity (Ekuni et al., 1998) and structural studies have indicated that both the N- and C-terminal are important for the interaction with the δ-subunit and the ε-subunit.

Based on these reports it was decided to fuse the HAT-tag to the N-terminal of the α, β and γ. The choice of the HAT-tag over the more traditionally used HIS_{6-10} tags was mainly based on the report that this HAT provides additional solubilisation effects when fused to proteins and in addition to this, also provides binding at slightly lower pH as compared to HIS_6-tags (Clontechniques, july 1998, Volume XIII(3): 27-28). However, as most reports dealing with *in vivo* reconstructions of the F_1-ATPase reported purification of native γ-subunits using extraction from SDS-PAGE gels as the means of purification, it was decided to include a native γ-subunit in the study. As overexpressed γ-subunit is found as inclusion bodies, purification of native γ subunit was performed by a simple washing procedure (see chapter 4).

Tagging of the delta and epsilon subunits

As was the case with the α, β and γ it has been reported that successful expression and complex reconstruction using both the δ- and ε-subunit n-terminally fused to the 26 kDa GST tag has been achieved (Shin et al., 1996). Detailed investigations of the structure of these subunits has revealed that despite the importance of the N-terminal as the physical link to other subunits truncations appeared to have little effect on activity of expressed complexes (Ni et al., 2004; Shi et al., 2001). On the other hand Keis et al. (2006) found that truncation of the N-terminal of the ε resulted in loss of activity and similar results were reported by Ni et al. (2004) for the δ-subunit.

The choice of the tag used in connection with δ- and ε-subunit was based on several considerations. Firstly the availability of magnetic supports naturally played a role. However, the choice of the Maltose Binding Protein tag (MBP) and Streptavidin were also intended, together with the HAT-tag used for the α, β and γ, to form a wide selection of binding options. In addition to this, the ability of MBP tag to serve as a favourable

fusion partner promoting solubilisation also contributed to the choice (Sørensen et al., 2003). Using the streptavidin/biotin affinity system the commonly used setup is based on streptavidin being coupled to the support, while biotin is attached to the target protein. However, this approach makes it very difficult to control the orientation of the adsorbed protein due to the relatively crude biotinylation coupling chemistries available. The ability to control the physical orientation of the protein, however is much easier achieved when using biotin coupled supports while including the streptavidin domain in the fused constructs. Primers used in connection with PCR reactions carried out as part of preparations of the F_1-ATPase subunits are listed in table C1.1.

Primer ID	Sequence (5' → 3')	Note
HAT-Fw	TATGAAGGATCATCTCATCCACAATGTCCACAAAGAGGAGCACGCTCA TGCCCACAACAAGGAGAACCTCTACTTTCAGGGTGCG	*HAT (Sence), AcTEV*
HAT-Rv	CGGCCGCACCCTGAAAGTAGAGGTTCTCCTTGTTGTGGGCATGAGCGT GCTCCT CTTTGTGGACATTGTGGATGAGATGATCCTTCA	*HAT (non-sense), AcTEV*
HATA-Fw	AAAAAAGCGGCCGCACAACTGAATTCCACCGAAATCAG	*NotI*
A-Rv	TCTACTGGATCCTTACCAGGATTGGGTTGCTT	*BamHI*
HATB-Fw	AAAAAAGCGGCCGCAGCTACTGGAAAGATTGTCCAG	*NotI*
B-Rv	TCTACTGGATCCTTAAAGTTTTTTGGCTTTTTCCACAG	*BamHI*
HATG-Fw	AAAAAAGCGGCCGCAGCCGGCGCAAAAGAGATACG	*NotI*
G-Fw	AAAAAACATATGATGGCCGGCGCAAAAGAGATA	*NdeI*
G-Rv	TCTACTGGATCCTTAAACCGCGGCGGCCCC	*BamHI*
MBPSA-Fw	AATAGTCATATGATGAAAATCGAAGAAGGTAAACTGG	*NdeI*
MBPSA-Rv	AACCCTGAAAGTAGAGGTTCTCCGAGTGGTGGTGGTGGTGGTGC	*Overlap*
D-Fw	GGAGAACCTCTACTTTCAGGGTTCGTCTGAATTTATTACGGTAGCTCG	*Overlap, AcTEV*
D-Rv	TTTTTTGGATCCCTATTAAGACTGCAAGACGTCTG	*BamHI*
E-Fw	GGAGAACCTCTACTTTCAGGGTTCGGCAATGACTTACCACCTGGA	*Overlap, AcTEV*
E-Rv	TTTTTTTGGATCCCTATTACATCGCTTTTTTGGTCAACTC	*BamHI*

Table C1.1: Primers used in this study. All primers are shown from the 5' end. The right column features included in the sequence: *HAT* refers to the sequence encoding the High Affinity Tag, while *AcTEV* refers to the sequence encoding the amino acid sequence recognized by the Tobacco Etch Virus protease (AcTEV) and *NdeI, NotI and BamHI* are restriction sites. Overlap describes a sequence severing as linker and overlap, and the sequence also includes the *AcTEV* site.

Restriction analysis of the prepared plasmid

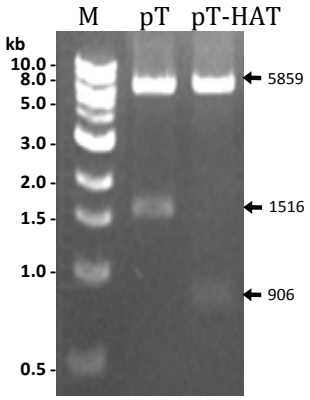


Figure C1.1: EtBr stained agarose gel displaying a RFLP analysis of pTwin1 (pT) and pTwin-HAT. The analysis was performed using *NdeI* and *BamHI. Restriction maps are available later in this appendix.*

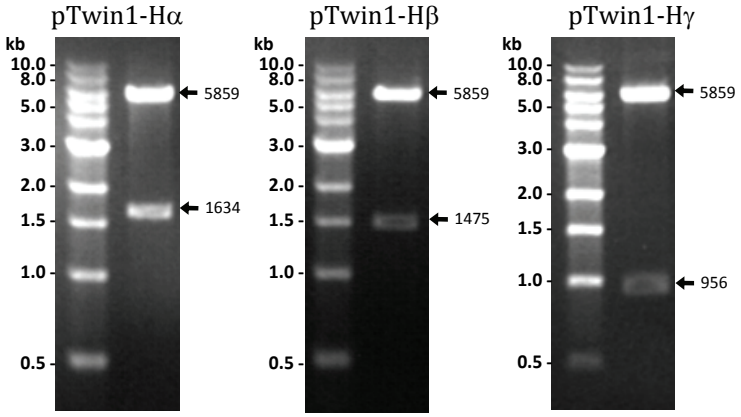


Figure C1.2: EtBr stained agarose gel showing the result of a RFLP analysis of the plasmids carrying the HAT tag ATPsynthase subunits α, β and γ. The analysis was performed as a double digest using *NdeI* and *BamHI.* Restriction maps are available later in this appendix.

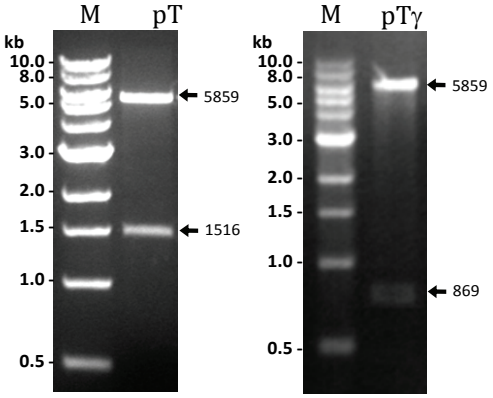


Figure C1.3: EtBr stained agarose gel displaying a comparable RFLP analysis of pTwin1 (pT) and pTwin-HAT(pTγ). The analysis was performed using *NdeI* and *BamHI.* Restriction maps are available later in this appendix.

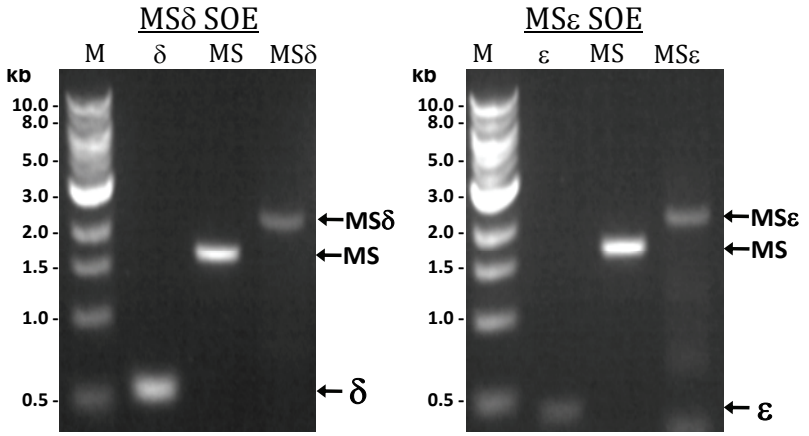


Figure C1.4: EtBr stained agarose gels showing the result of SOE by PCR performed between the MBP-SA encoding fragment and subunits δ or ε. Lanes marked with δ, ε or MS contains the purified fragments, while lanes marked MSδ or MSε shows the fused PCR products.

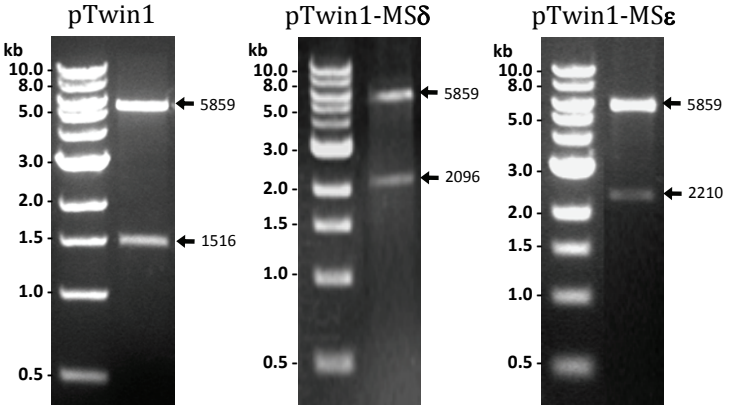
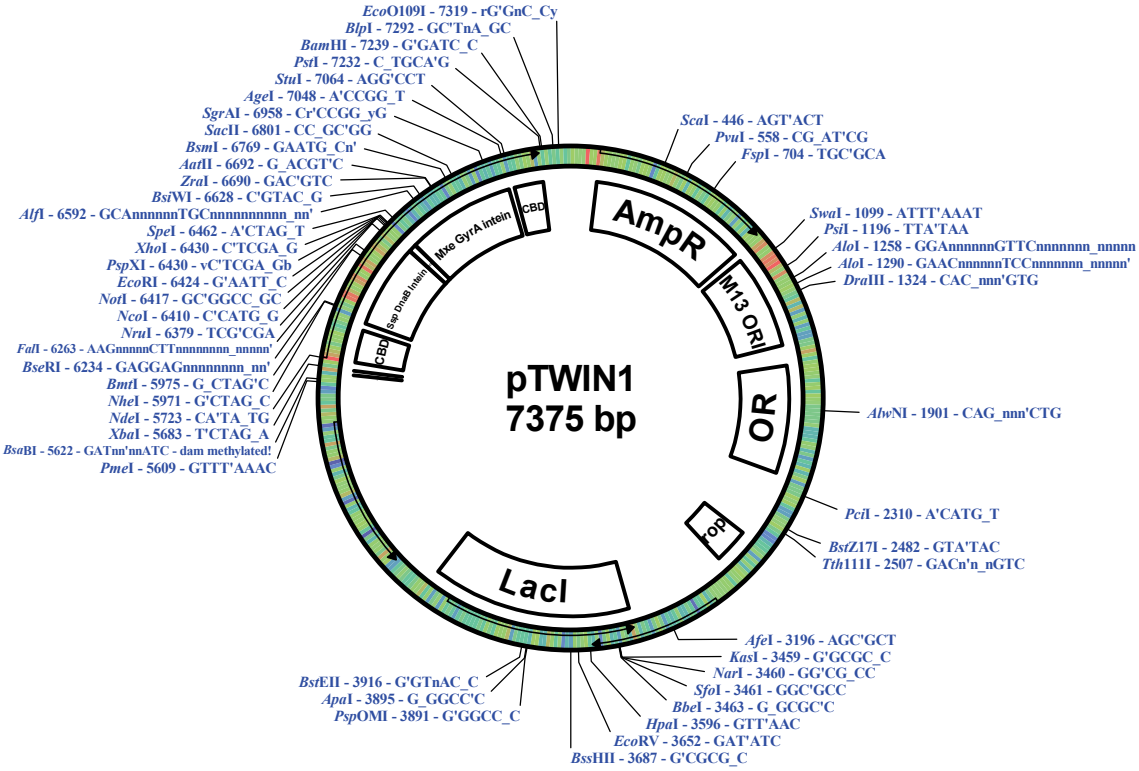
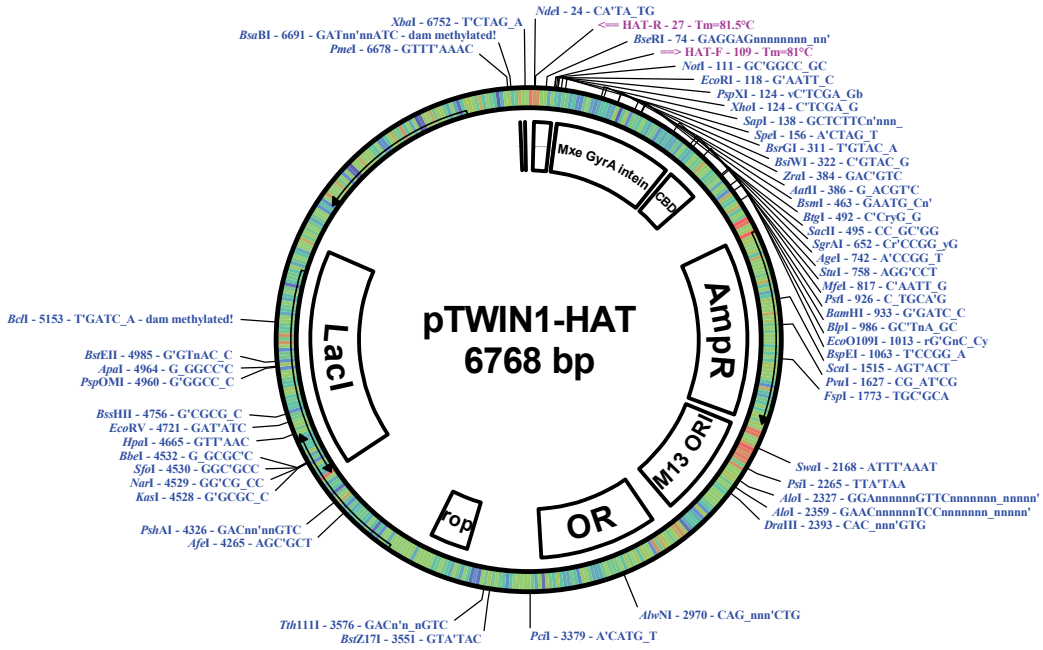


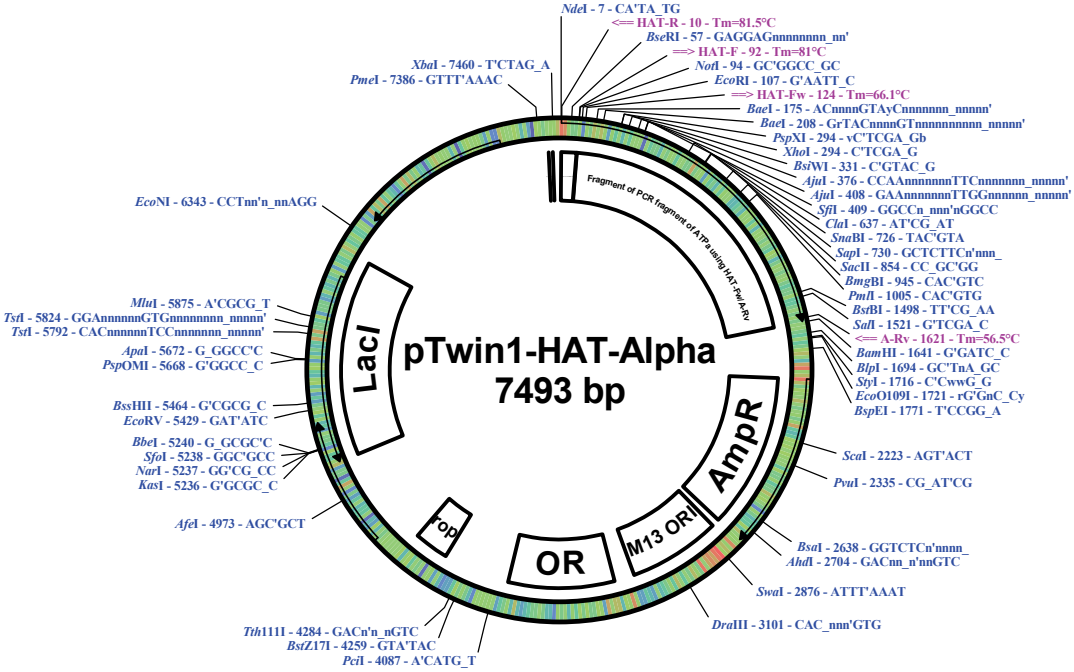
Figure C1.5: EtBr stained agarose gel showing RFLP analysis of the cloned fusions between the MS fragment and δ or ε encoding DNA fragment. The analysis was performed using *NdeI* and *BamHI.* Restriction maps are available later in this appendix.

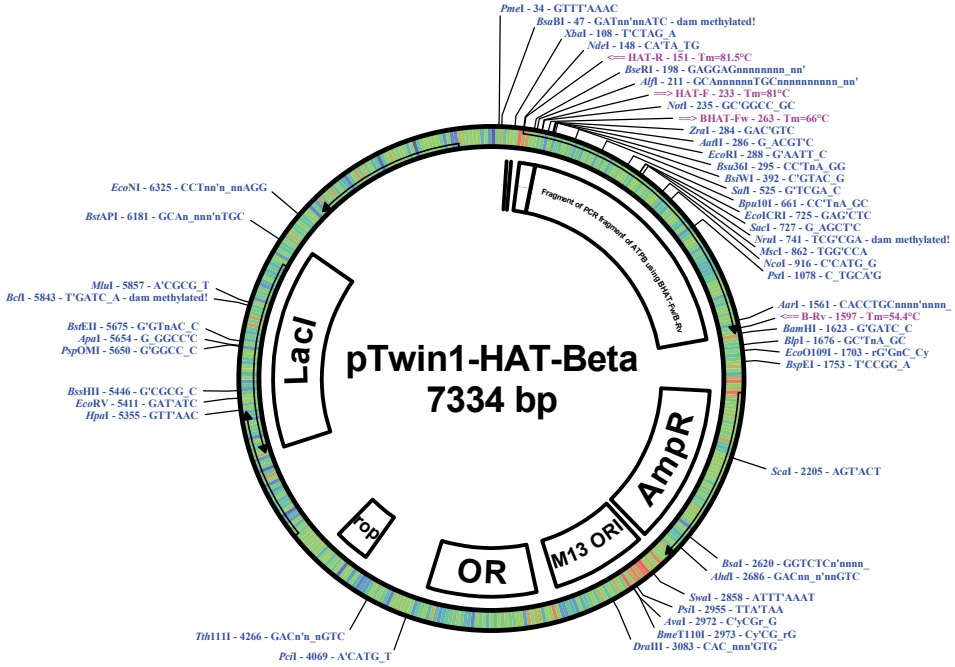
Restriction maps of pTwin1 and pTwin1-HAT



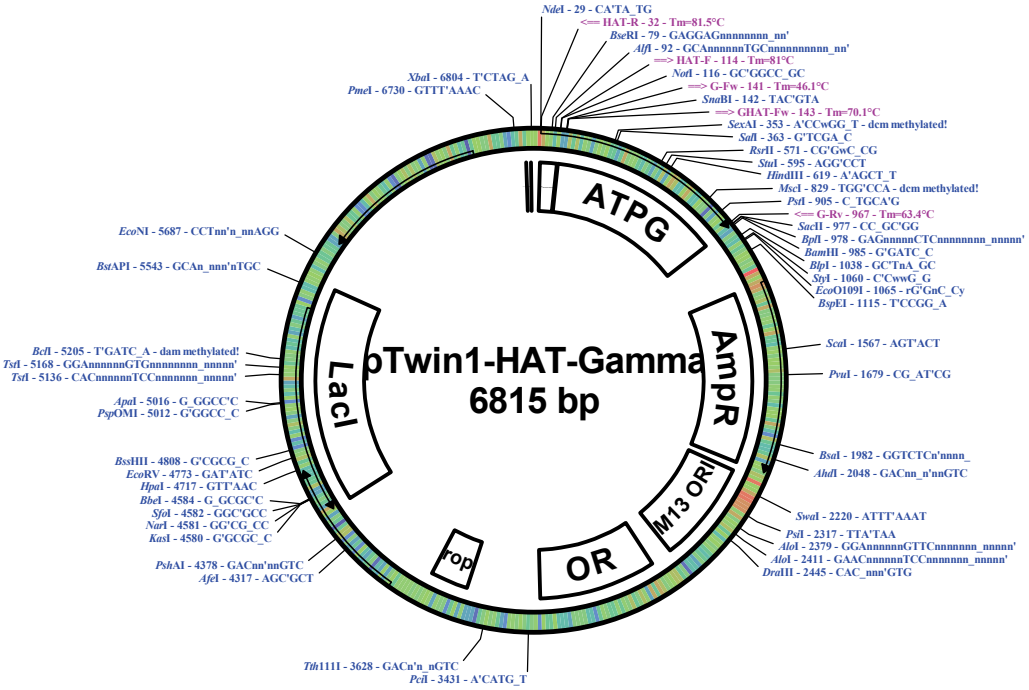


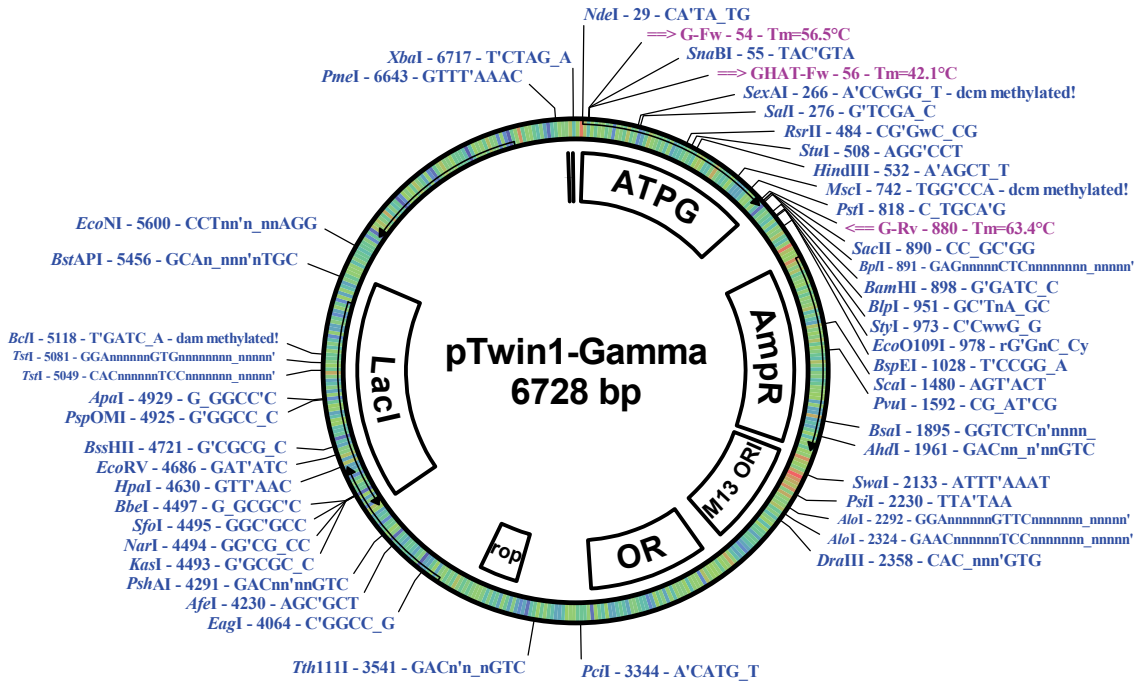
Restriction maps of pTwin1-Hα and pTwin1-Hβ



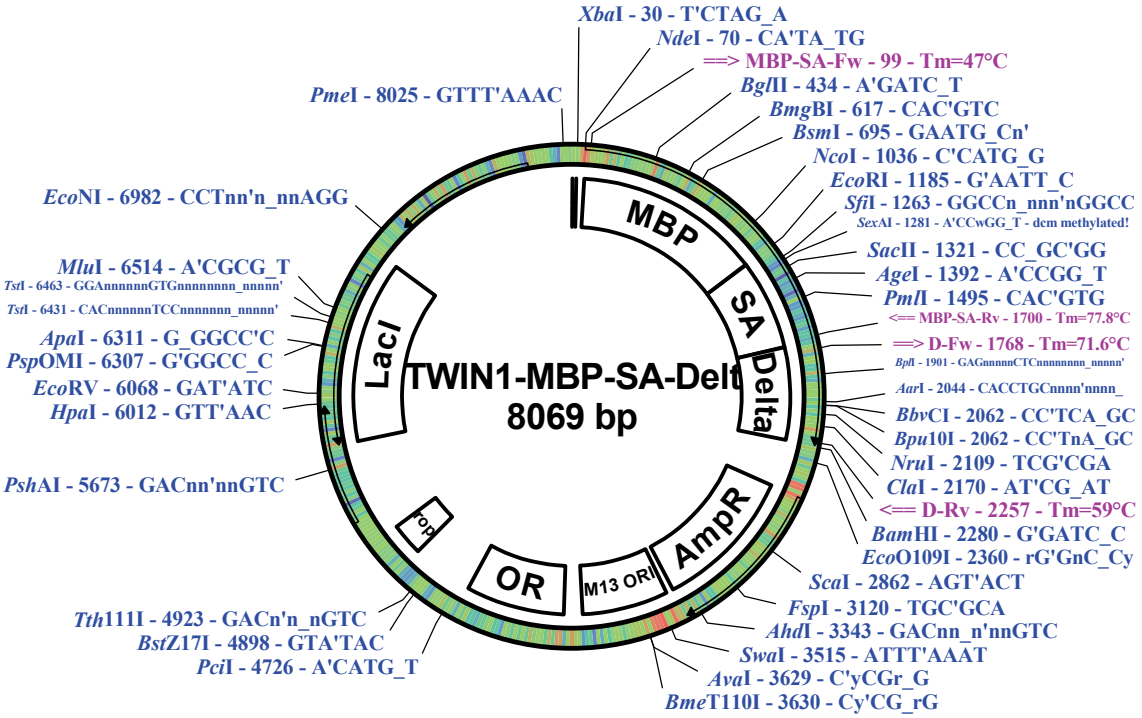


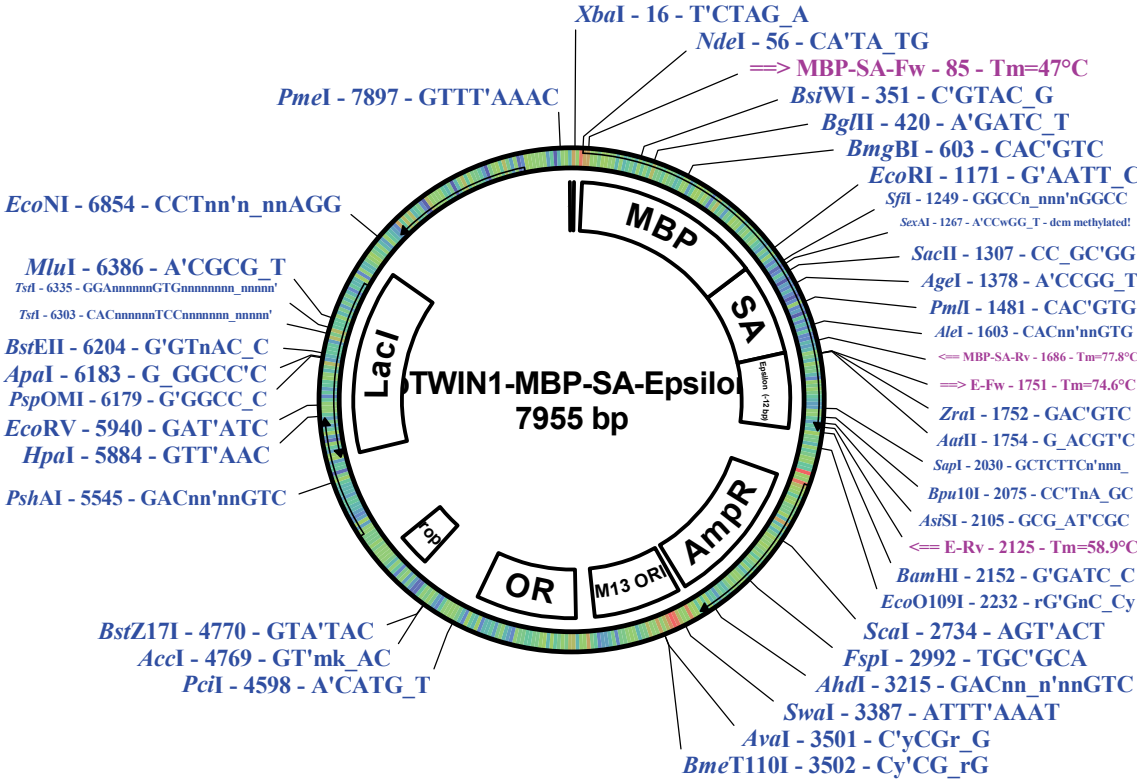
Restriction maps of pTwin1-Hγ and pTwin1-γ





Restriction maps of pTwin1-MSδ and pTwin1-MSε





6.1.1 Chromatograms from the purification of the overexpressed denatured F_1-ATPase subunits

HAT-Alpha (Hα)

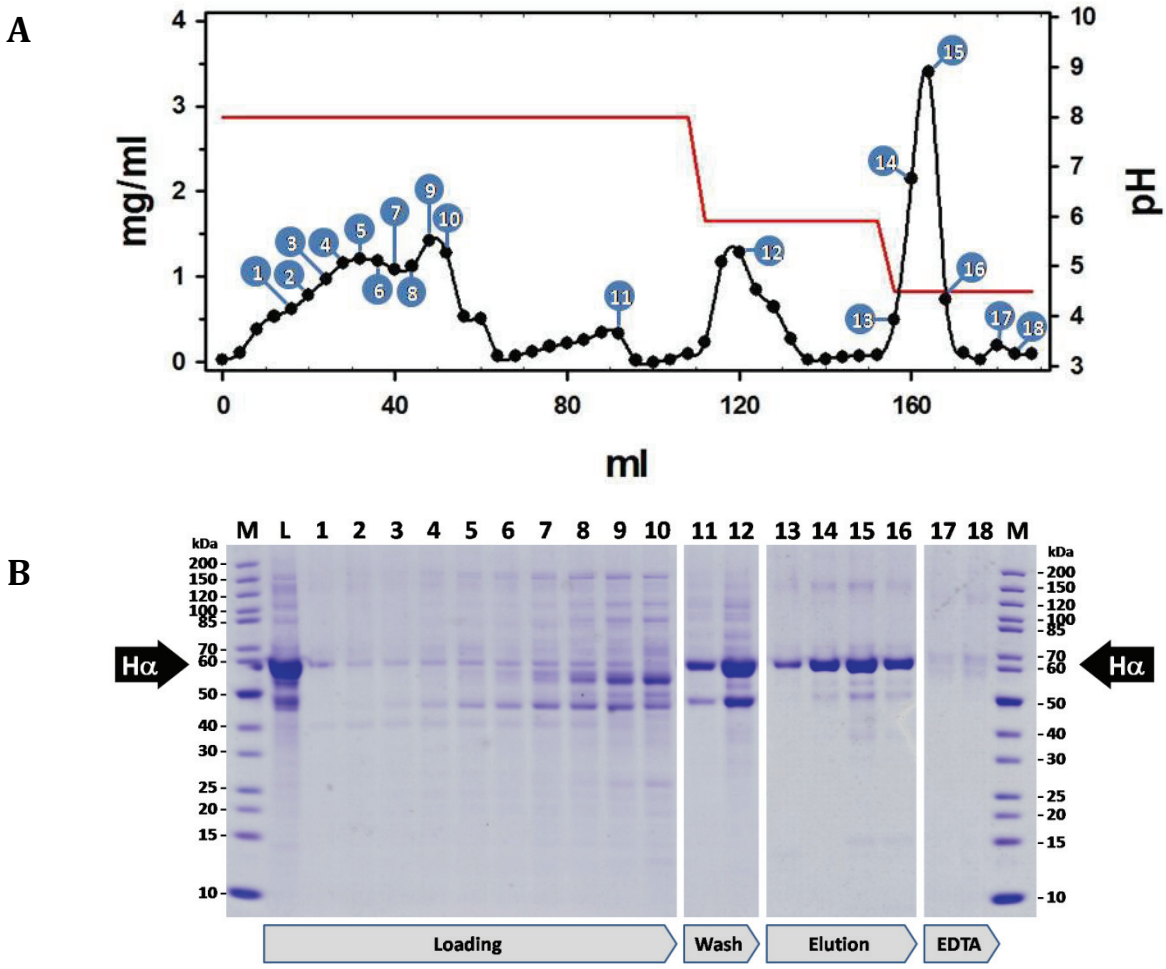


Figure C1.6: Purification of the HAT-α subunit using Nickel charged Sepharose Fast flow. (A) Chromatogram prepared by measurement of the protein contents in the isolated fractions. The corresponding pH values are shown in red. **(B)** Reduced SDS-PAGE analysis showing the contents of selected samples. The number noted at the top of the gels corresponds to the numbers noted in the chromatogram. Black arrows indicate the position of the HAT-α subunit (58.6 kDa).

HAT-Beta (Hβ)

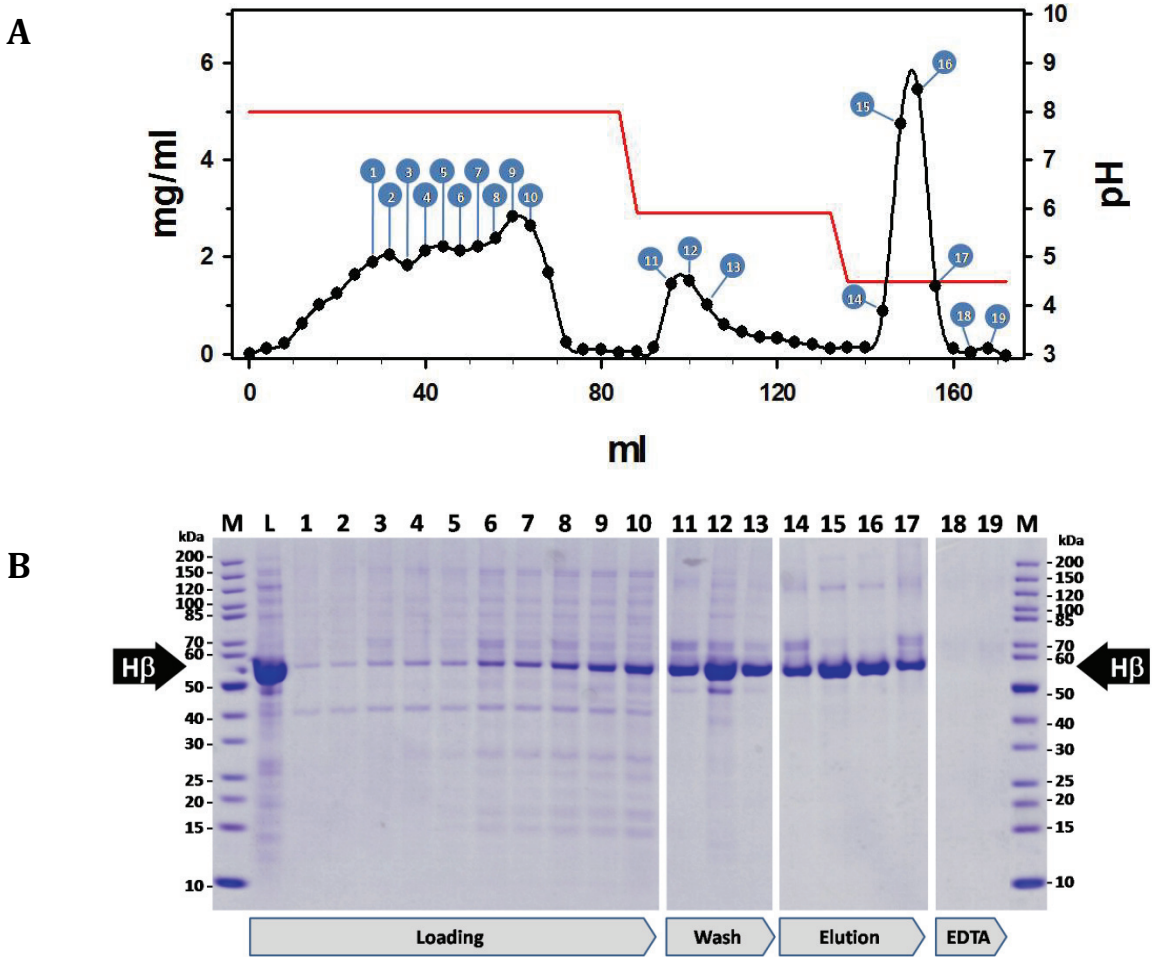


Figure C1.7: Purification of the HAT-γ subunit using Nickel charged Sepharose Fast flow. (A) Chromatogram prepared by measurement of the protein contents in the isolated fractions. The corresponding pH values are shown in red. **(B)** Reduced SDS-PAGE analysis showing the contents of selected samples. The number noted at the top of the gels corresponds to the numbers noted in the chromatogram. Black arrows indicate the position of the HAT-γ subunit (53.8 kDa).

HAT-Gamma (Hγ)

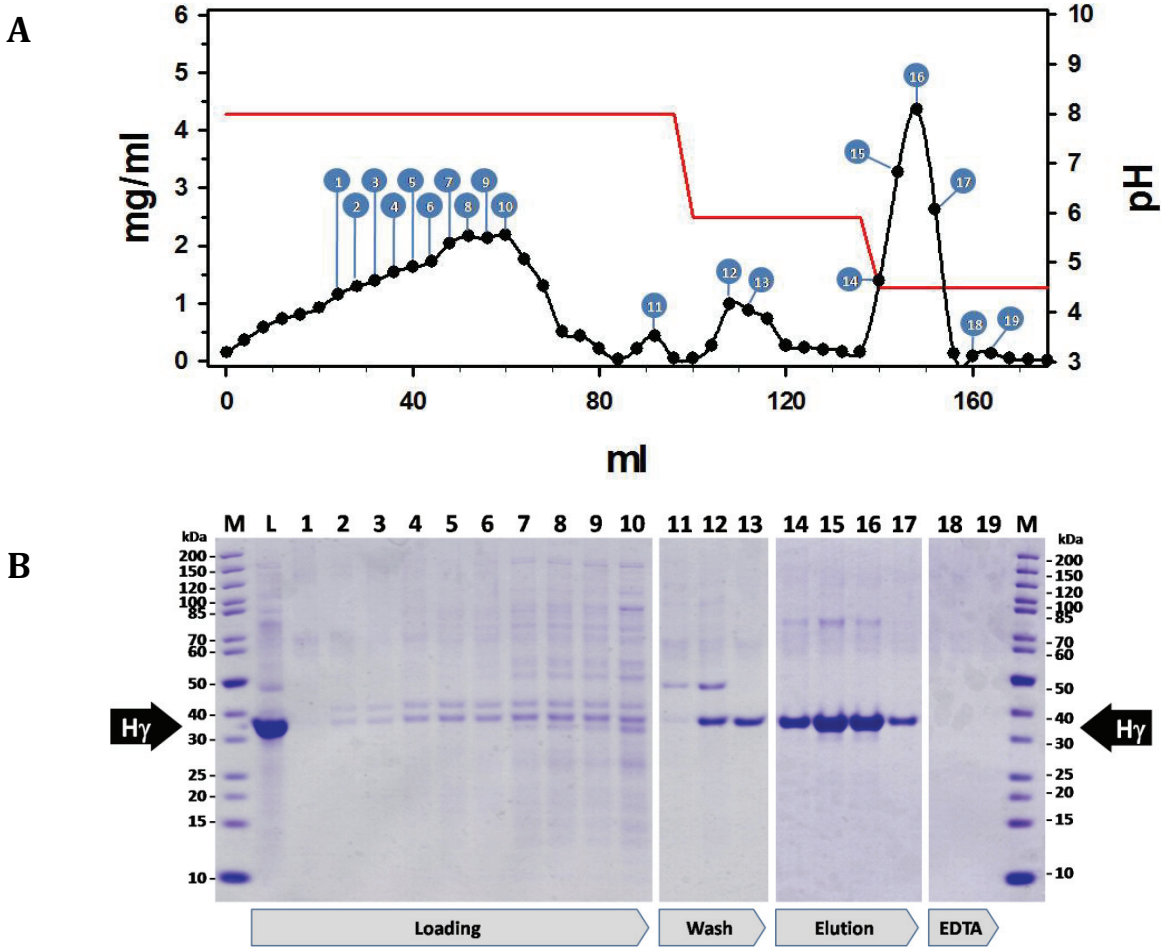


Figure C1.8: Purification of the HAT-γ subunit using Nickel charged Sepharose Fast flow. (A) Chromatogram prepared by measurement of the protein contents in the isolated fractions. The corresponding pH values are shown in red. **(B)** Reduced SDS-PAGE analysis showing the contents of selected samples. The number noted at the top of the gels corresponds to the numbers noted in the chromatogram. Black arrows indicate the position of the HAT-γ subunit (34.9 kDa).

MBP-SA-Delta (MSδ)

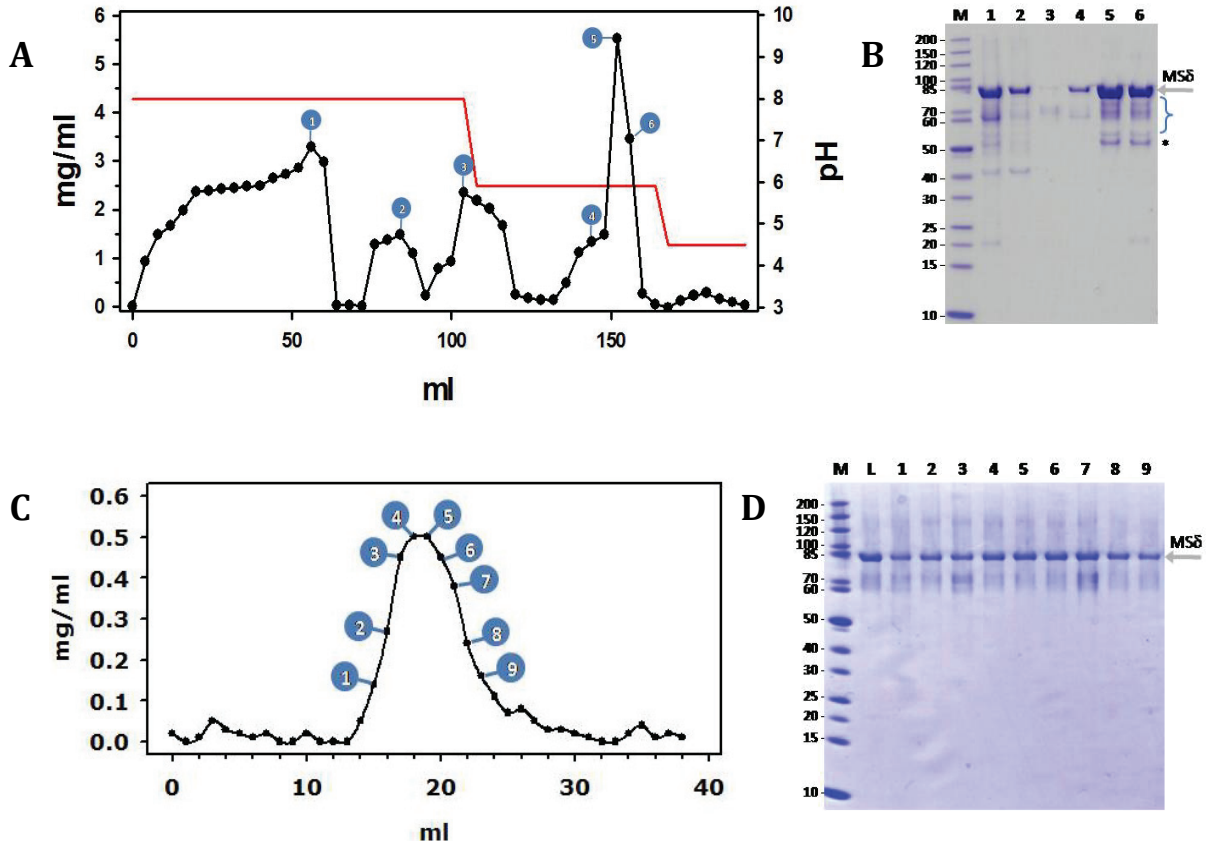


Figure C1.9: Purification of MSδ subunit using Nickel charged Sepharose Fast flow and size exclusion chromatography. (A) Chromatogram prepared by measurement of the protein contents in the isolated fractions in IMAC. The corresponding pH values are shown in red. **(B)** Reduced SDS-PAGE analysis showing the contents of selected samples origination from the IMAC procedure. The number noted at the top of the gels corresponds to the numbers noted in the chromatogram. **(C)** Chromatogram from the size exclusion chromatogram obtained using Sepharose S-300 HR (Amersham Biosciences), while **(D)** shows the result of SDS-PAGE analysis. The numbers noted in the chromatogram corresponds to the lanes shown in the SDS-PAGE analysis. The arrows indicate the position of the MSδ subunit (79.6 kDa).

MBP-SA-Delta (MSε)

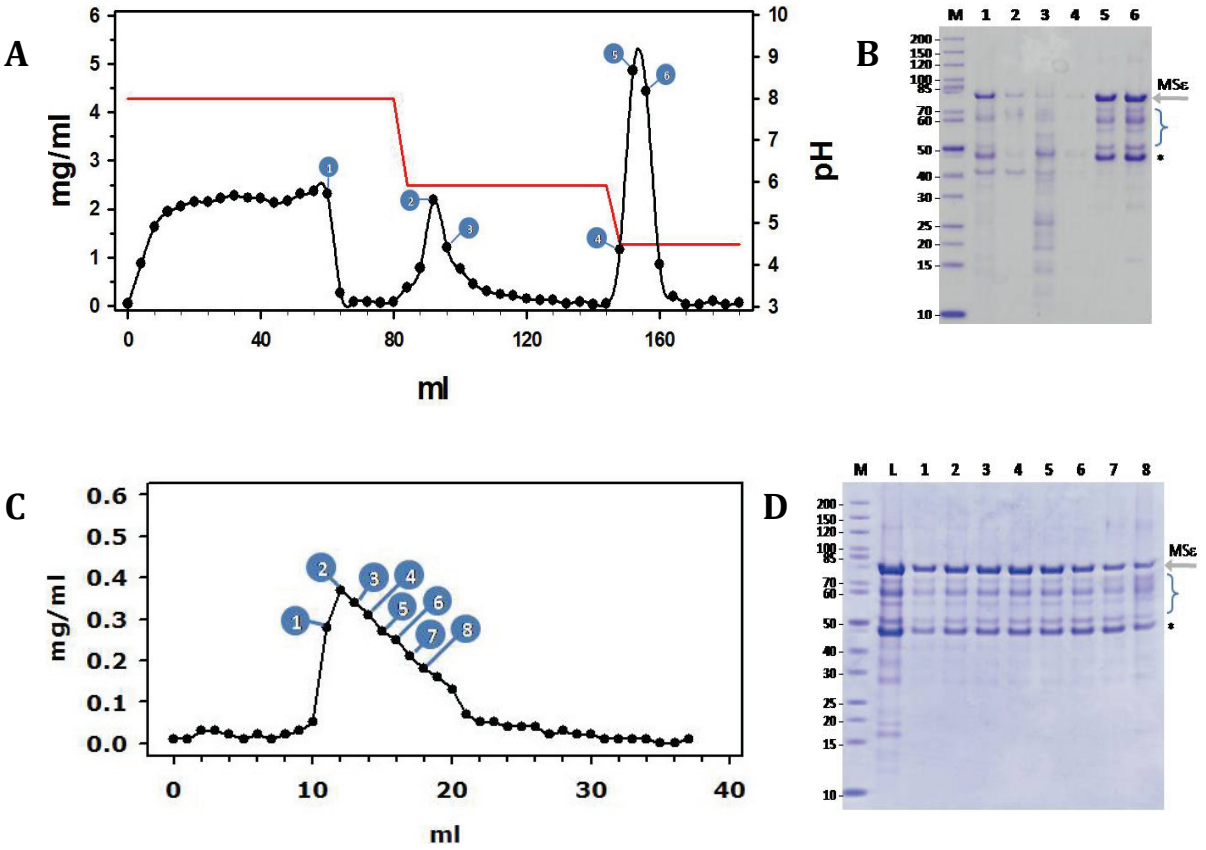


Figure C1.10: Purification of MSε subunit using Nickel charged Sepharose Fast flow and size exclusion chromatography. (A) Chromatogram prepared by measurement of the protein contents in the isolated fractions in IMAC. The corresponding pH values are shown in red. **(B)** Reduced SDS-PAGE analysis showing the contents of selected samples origination from the IMAC procedure. The number noted at the top of the gels corresponds to the numbers noted in the chromatogram. **(C)** Chromatogram from the size exclusion chromatogram obtained using Sepharose S-300 HR (Amersham Biosciences), while **(D)** shows the result of SDS-PAGE analysis. The numbers noted in the chromatogram corresponds to the lanes shown in the SDS-PAGE analysis. The arrows indicate the position of the MSε subunit (75.3 kDa).

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Subunit gamma-green fluorescent protein fusions are functionally incorporated into mitochondrial F1F0-ATP synthase, arguing against a rigid cap structure at the top of F1.
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Structural characterization of the interaction of the delta and alpha subunits of the Escherichia coli F1F0-ATP synthase by NMR spectroscopy.
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6.4 Appendix D: : Käppler et al. / Peer reviewed paper

ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

In Situ Magnetic Separation for Extracellular Protein Production

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ABSTRACT: A new approach for in situ product removal from bioreactors is presented in which high-gradient magnetic separation is used. This separation process was used for the adsorptive removal of proteases secreted by *Bacillus licheniformis*. Small, non-porous bacitracin linked magnetic adsorbents were employed directly in the broth during the fermentation, followed by in situ magnetic separation. Proof of the concept was first demonstrated in shake flask culture, then scaled up and applied during a fed batch cultivation in a 3.7 L bioreactor. It could be demonstrated that growth of *B. licheniformis* was not influenced by the in situ product removal step. Protease production also remained the same after the separation step. Furthermore, degradation of the protease, which followed first order kinetics, was reduced by using the method. Using a theoretical modeling approach, we could show that protease yield in total was enhanced by using in situ magnetic separation. The process described here is a promising technique to improve overall yield in bio production processes which are often limited due to weak downstream operations. Potential limitations encountered during a bioprocess can be overcome such as product inhibition or degradation. We also discuss the key points where research is needed to implement in situ magnetic separation in industrial production.

Biotechnol. Bioeng. 2009;102: 535–545.

KEYWORDS: magnetic separation; downstream processing; protein purification; bioseparation; in situ-product-removal

Introduction

Bioprocess intensification has often been focused on decreasing the number of bioseparation steps. Much work has been done on the use of direct capture methods such as expanded bed adsorption (EBA) and high gradient magnetic fishing (HGMF) to recover the product directly from a crude fermentation broth (Hubbuch et al., 2001). These techniques can bypass the need for several purification steps such as filtration, centrifugation or extraction before packed bed chromatography is performed.

Another approach to intensify product recovery is to integrate product formation and recovery; this can be achieved by in situ product removal (ISPR) in which the product is separated during the bioprocess (Freeman et al., 1993; Stark and von Stockar, 2003). In addition to the reduced number of process steps it is also beneficial that the interaction of the accumulated product with the system (e.g., product inhibition) is reduced. Furthermore, losses due to uncontrolled product damage which can arise from reactions with substances present in the broth are diminished. These substances can be proteases which can digest themselves as well as other targets in the broth.

There are many different types of separation technology for ISPR such as evaporation, extraction, permeation (e.g., membrane filtration), precipitation or adsorption. For proteins, adsorptive and extractive methods have been used for ISPR (Millitzer et al., 2005). The technique of adsorption has the advantage of being selective and also flexible with regard to the surface and ligand chemistries that are available. In principle these functionalizations can be adapted from those used in chromatography. Both EBA and HGMF are adsorptive bioseparation methods designed for application in crude biofeedstocks, raising the possibility of adapting them to in situ product recovery. However, EBA is a type of fluidized bed chromatography and suffers from a well known problem of adsorbent aggregation during crude feedstock processing. This upsets the delicate fluidization of the bed, which limits in situ application during a fermentation. On the other hand, HGMF relies on using a batch adsorption step in which micron-sized non-porous magnetic adsorbents bind the target and can subsequently be

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Contract grant sponsor: German Academic Exchange Service (DAAD) program RISE

removed rapidly from the broth by magnetic separation (Franzreb et al., 2006; Oberteuffer, 1974; Safarik et al., 1995; Setchell, 1985). Since the magnetic adsorbents are non-porous with respect to proteins (O'Brien et al., 1996), and have highly specific surfaces they are not prone to fouling. A batch adsorption step is easy to implement during a fermentation, requiring that the adsorbents simply be added to the bioreactor. High gradient magnetic separation technology is scaleable and in principle could be easily integrated in a recycle loop in a bioreactor for the rapid recovery of loaded magnetic adsorbents. A preliminary work was recently reported showing the stabilization of bovine serum albumin during a fermentation, following protease removal with magnetic adsorbents (Ottow et al., 2007).

This article introduces the concept of using magnetic separation as an in situ product removal tool defined here as in situ magnetic separation (ISMS). This has been examined in a fermentation process carried out both in shake flasks and bioreactor. *Bacillus licheniformis* was cultivated which produces extracellular proteases that could be removed using a magnetic separation process.

Materials and Methods

Materials

B. licheniformis 1969 was obtained from the DSMZ (Braunschweig, Germany).

Subtilisin from *B. licheniformis*, azocasein, and the chemicals 3-aminopropyltriethoxysilane (440140), glycerol, glacial acetic acid, KH_2PO_4, NaOH, NaCl, methanol, glutaraldehyde (66403), NaCl, $NaBH_4$, divinylsulfone (DVS) (V3700), bacitracin (B0125-1250 KU), Na_2MoO_4 and disodium succinate were obtained from Sigma-Aldrich (St. Louis, MO). $FeCl_2$ and $FeCl_3$ were obtained from Riedel-de Haën (Seelze, Germany). Glucose, Na-citrate, $CaCl_2$, Na_2CO_3, NaCl, propylene glycol and disodium tetraborate were purchased from Roth (Karlsruhe, Germany); $(NH_4)_2SO_4$, $MgSO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4 \cdot 7\ H_2O$, $ZnSO_4 \cdot 7\ H_2O$, NaH_2PO_4, $MnCl_2 \cdot 4\ H_2O$ and $CuCl_2 \cdot 2\ H_2O$ from Merck (Darmstadt, Germany).

The electromagnet was obtained from Steinert (Cologne, Germany), magnetic matrices consisting of wires made of stainless steel (AISI 430; X6Cr17) were from Haver&Boecker (Oelde, Germany). NdFeB permanent magnets were purchased from Webcraft (Uster, Switzerland) and the "Chemagic" magnetic stand for adsorbent separations in test tubes was obtained from Chemagen (Baesweiler, Germany).

Preparation of Bacitracin-Linked Magnetic Adsorbents

Bacitracin-linked superparamagnetic adsorbents were produced in order to separate alkaline serine proteases from the crude fermentation broth of *B. licheniformis*. The methods of Hoffmann (2003) and O'Brien et al. (1996, 1997) were employed. Briefly, iron chloride salts were precipitated in aqueous sodium hydroxide solution at pH 11–12 (Misawa et al., 1974). The molar ratio of Fe(III)/Fe(II) was 2 and the molar excess of sodium hydroxide 2.5. The composition of the precipitates produced is known to be very heterogeneous and includes magnetite, which is the most desired component and has a high magnetization, as well as many other iron oxides with much lower magnetization, for example, hematite (Cornell and Schwertmann, 1996). The nano-sized precipitates were then silanized with 3-aminopropyltriethoxysilane at 110°C (Hubbuch and Thomas, 2002). Silanization aggregates the precipitates to micron sized particles and helps protect the iron oxide core from oxidation processes. The free amino groups of the silane-network were coupled with polyglutaraldehyde (O'Brien et al., 1996, 1997) to create superparamagnetic micron- or submicron-sized beads with high magnetization and no magnetic memory. In the next step the polyglutaraldeyde coating was activated by DVS: A thin layer of polyglutaraldehyde can easily be activated by DVS (Halling and Dunnill, 1979). Finally bacitracin was coupled to the DVS-activated beads by standard methods (Hermanson et al., 1992). Bacitracin belongs to the group of cyclic peptide antibiotics with a molecular weight of 1.42 kDa and is capable of specifically binding alkaline serine proteases, for example, Subtilisin Carlsberg (EC 3.4.21.62), which has a molecular weight of about 28 kDa (Vitkovic and Sadoff, 1977). The synthesized particles were then characterized.

Particle diameter determined from q_3-distributions (x_{50}) (with Coulter Multisizer II from Beckmann-Coulter, Fullerton, CA) was found to be 7.2–17.3 μm (without ultrasound treatment), the saturation magnetization (M_S) was 21 $A\,m^2\,kg^{-1}$, and the remnant magnetization (M_R) was 0.025 $A\,m^2\,kg^{-1}$. These were determined using an alternating gradient magnetometer (Micromag 2900 from Princeton, NJ). The binding characteristics of the adsorbents were determined using batch adsorption studies using commercially obtained subtilisin from *B. licheniformis* (Sigma-Aldrich). A 50 mM tris buffer, pH 7.5, was used together with incubation with shaking at room temperature for 30 min at 300 RPM. The isotherm found is shown in Figure 1 and when fitted with the Langmuir model (using Origin software, OriginLabs, Northampton, MA) the maximum binding capacity (Q^*_{max}) was 0.298 $g\,g^{-1}$ and apparent dissociation constant (K_d) was 0.32 μM, values which fall well within the categories defined by Franzreb et al. (2006, 2007) for acceptable magnetic adsorbents. Although the Langmuir model does not provide a perfect fit (r^2 value of 0.8), the isotherm shows what is to be expected for a strong, highly specific affinity interaction, namely an extremely steep initial slope followed closely by a plateau region. The data shows no evidence of a subsequent upward trend and it can thus be concluded that multilayer binding was of little consequence under the conditions used. The initial slope of the isotherm suggests that the working capacity is as high as 0.1–0.15 $g\,g^{-1}$ adsorbents.

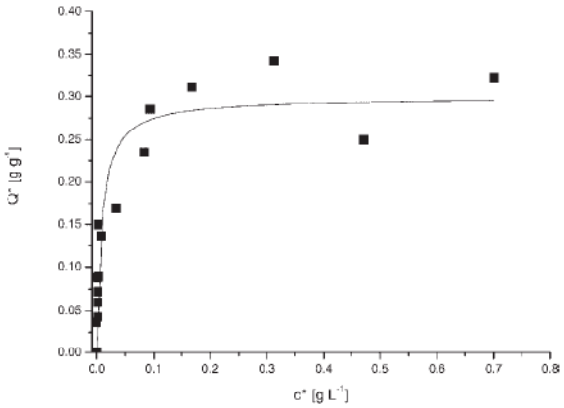

Figure 1. Adsorption isotherm for the binding of subtilisin to bacitracin functionalized magnetic particles. A 50 mM Tris-buffer at pH 7.5 was used. Solid line shows the fit to the Langmuir model and gives $K_d = 3.2 \times 10^{-7}$ mol L^{-1}; $Q^*_{max} = 0.298$ g g^{-1}.

Cultivation Procedure

Cultivations were performed with *B. licheniformis* (DSMZ 1969) either on complex medium (shake flask experiments) or on a synthetic medium (bioreactor experiments). The complex medium was composed of (g L^{-1}): peptone 5, meat extract 3, glucose 30. The synthetic medium consisted of (g L^{-1}): Na-citrate 1; $(NH_4)_2SO_4$ 10; KH_2PO_4 3.7; K_2HPO_4 6.8; $MgSO_4$ 0.2; glucose 30; trace elements (mg L^{-1}): $CaCl_2$ 2.2; Na_2MoO_4 0.24; $FeSO_4 \cdot 7H_2O$ 5.2; $ZnSO_4 \cdot 7H_2O$ 7.2; $MnCl_2 \cdot 4H_2O$ 10; $CuCl_2 \cdot 2H_2O$ 0.85. The feed added during fed-batch cultivations consisted of (g L^{-1}): $(NH_4)_2SO_4$ 4.8; glucose 527, trace elements (fourfold concentration of synthetic medium see above). The ammonium sulfate concentration was calculated based on the biomass concentration expected and the culture stoichiometry to ensure nitrogen limitation in the fed-batch phase. The stoichiometry of the fermentation was determined in preliminary batch experiments using synthetic medium in the bioreactor. These experiments gave a yield coefficient of $Y_{X,N}$ 3.6 $g_{Biomass}/g_{ammonium}$. Sterilization of reactors, tubes and shake flasks was performed by autoclaving. Here glucose was treated separately.

The innoculum for all cultivations consisted of an overnight culture of *B. licheniformis* at 37°C. Between 4 and 10 mL of the culture was then used to innoculate 200 mL of medium in a 0.5 L shake flask. With this shake flask small scale experiments were carried out. Alternatively, the shake flask culture was grown for 20 h and then all of it was used to innoculate a bioreactor. Shake flasks were cultivated at 37°C on a shaker with 170 RPM.

Bioreactor cultivations were performed in a 3.7 L bioreactor from Bioengineering (Wald, Switzerland) which was equipped with a pH-electrode from Bioengineering (Wald, Switzerland) and a pO_2-electrode from Mettler-Toledo (Giessen, Germany). Stirrer speed was pO_2-controlled between 400 and 1,000 rpm; setpoint for pO_2-saturation was 20%. The off gas was analysed continuously using a Maihak analyser (Reute, Germany) and allowed measurements of the oxygen and carbon dioxide content. The reactor was controlled by a program based on Labview from National Instruments (Austin, TX). Bioreactor cultivations had an initial volume of 2.25 L and were inoculated from shake flask cultures grown using synthetic medium. During the fed batch phase a constant flow rate of 6 or 25 mL h^{-1} was added, as described in the text.

In Situ Magnetic Separation Studies

Shake Flask ISMS

After inoculation, the culture was allowed to grow and samples were taken periodically for analysis. After 18 h significant amounts of proteases had been produced and at this time 30 mL of the culture was mixed with 35 mg of pre-sterilized magnetic adsorbents before the suspension was added to a second shake flask (250 mL) (Fig. 2). Adsorption of proteases took place over a 20 min period, in which the shake flask was incubated with shaking at 37°C and 170 RPM. Subsequently, a permanent magnet held to the bottom of the flask was used (≤10 min) to separate the adsorbents before the broth was decanted back to a 250 mL shake flask (Fig. 2). The captured adsorbents could then be used for elution studies. Samples (1–2 mL) were taken several times during the procedure. To determine the effects of ISMS, a second shake flask was cultivated in the same way, without adding any adsorbents. When magnetic adsorbents

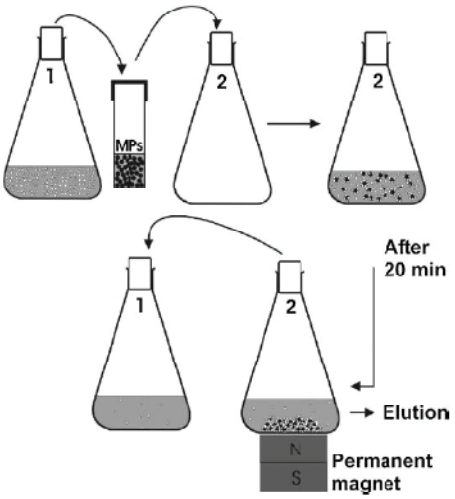

Figure 2. Procedure for conducting ISMS in shake flask cultivations. MPs, magnetic adsorbents.

were used, they were first sterilized by washing in 76% ethanol for at least 20 min. They were then magnetically captured using a bar magnet, excess ethanol decanted and they were washed three times in sterile water.

Bioreactor ISMS

The use of a simple permanent magnet for adsorbent separation is not possible when the ISMS process is performed on a larger scale. However, HGMS technology employing a magnetic filter and electromagnet can efficiently capture small magnetic adsorbents. Furthermore, a HGMS system can be established which is capable of completing further process steps, such as washing and elution of the magnetic particles. The process designed for the current work is depicted in Figure 3. In essence it consisted of a system of valves, tubes and pumps to allow adsorption of the proteases, recovery of the adsorbents and then adsorbent washing and elution of the captured proteases. Prior to use, all parts to come in contact with the fermentation broth were sterilized. This was done primarily by flushing the complete system with 76% ethanol for at least 30 min, followed by sterile water. The valve system was then connected to the bioreactor. At the appropriate time, the biosuspension (2.25 L) was pumped using peristaltic pump P1 (Ismatec, Glattbrugg, Switzerland) into the adsorption vessel (i.e., a glass bottle) which contained 4 g of sterile adsorbents and was shaken for 20 min to allow binding of the proteases. Using peristaltic pump P2 (Ismatec) the broth was then pumped through valve system V1 and the HGMS filter with the magnet switched on (HGMS filter is shown in Fig. 4), through the HGMS closed loop, and back to the bioreactor via valve system V2, after which the cultivation continued. Capture of the magnetic adsorbents took only ca. 10 min. The loaded magnetic adsorbents were thus removed from the broth and could be washed and eluted in the HGMS closed loop.

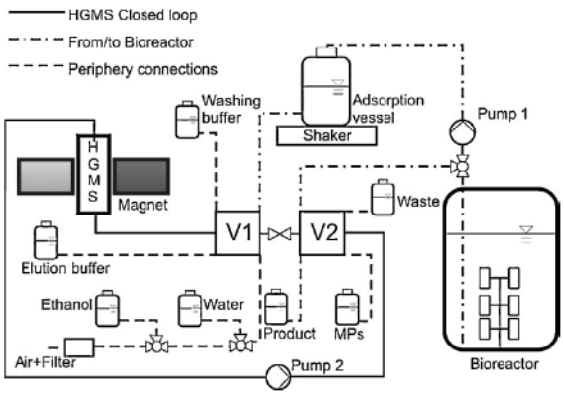

Figure 3. Schematic of the ISMS system integrated with a bioreactor. Liquids are moved using two peristaltic pumps (P1 and P2). Valve systems (V1 and V2) are used to control fluid flow in the system.

Wash and elution of the adsorbents was conducted in a similar way to that described previously for HGMF systems (Franzreb et al., 2006, 2007; Hoffmann et al., 2002; Hubbuch et al., 2001). Using pump P2, the HGMS closed loop was filled with the relevant buffer, the magnet switched off and the particles released into the closed loop by pumping with pump P2. After 1 h the magnetic field was switched on and the magnetic adsorbents captured, after which the buffer in the closed loop was collected. Subsequently the cycle could be repeated with a new wash or elution buffer. The composition of washing buffer was 0.1 M Tris, 0.02 M $CaCl_2$, pH to 7.5. The elution buffer contained 0.005 M succinate (di-sodium-salt), 0.05 M sodium tetraborate, 0.001 M $CaCl_2$, propylene glycol (50% (v/v)), pH 6. Each valve system, V1 and V2, consisted of four single, sterilisable and pneumatically operated diaphragm valves (purchased from Gemue, Ingelfingen-Criesbach, Germany). The direction of fluid-flow through each valve was pre-determined, and the whole procedure including the bioreactor performance was controlled by a Labview programme.

Analysis

Protease activity was measured using a colorimetric assay employing azocasein as substrate and the adsorbance at 420 nm was measured after 30 min (Charney and Tomarelli, 1947). A commercial subtilisin from Sigma-Aldrich was used to generate a standard curve of activity versus enzyme concentration to allow the protease concentration in the fermentation to be determined.

The rate of protease degradation was determined in synthetic medium (without glucose) pH 7.5 as well as in the supernatant of fermentation broth. The solutions under test were placed in a tube and incubated at 37°C. At various times a sample was removed, immediately placed on ice and then the activity determined using the azocasein assay.

Total protein content was determined using a modified Lowry assay kit (Peterson's modification) obtained from Sigma-Aldrich and was conducted according to the manufacturers instructions (Bensadoun and Weinstein, 1976; Lowry et al., 1951; Peterson, 1977).

During fermentations, glucose concentration was determined using the Glucose liquicolor GOD-PAP test kit by Human (Wiesbaden, Germany) (Trinder, 1969). Biomass concentration was measured by turbidimetry via OD at 550 nm and converted to biomass dry weight by using a standard curve. For all measurement methods, reproducibility of the analysis was examined and the standard deviation found to be 9% for protein measurements, 6% activity measurements, 9% for glucose measurements, and 10% for biomass measurements.

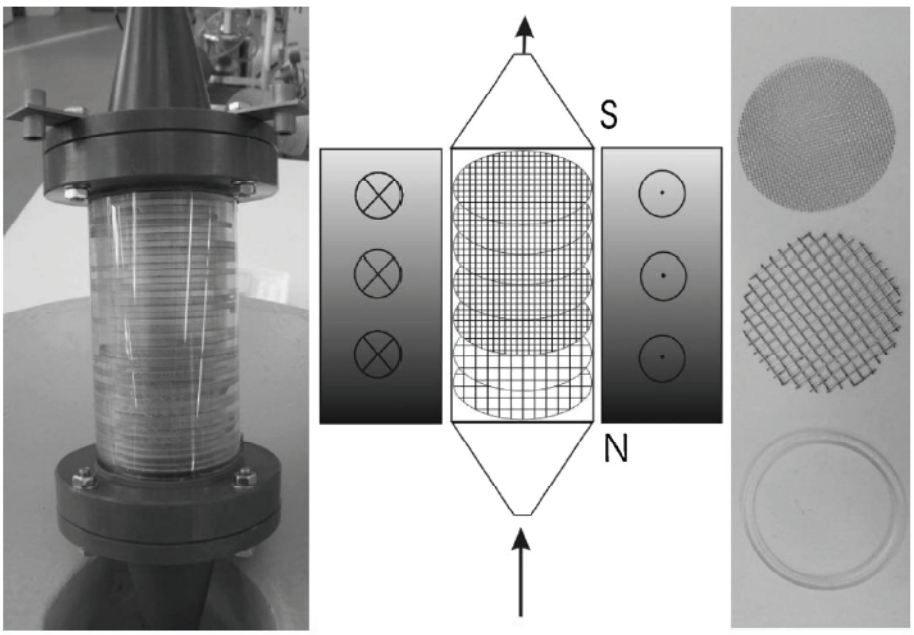

Figure 4. HGMS filter canister employed for the ISMS procedure depicted in Figure 3. Left hand picture: HGMS filter canister packed with filter meshes and ready for lowering into the electromagnet core that it is sitting above. Centre: Schematic of HGMS filter canister, and different filter mesh disks used, inside the electromagnet; arrows show fluid flow; outside is the electromagnet coil (electric current direction indicated within circles). Right hand picture: Types of 55 mm diameter filter mesh disks used inside the magnetic canister; bottom picture, spacer; middle picture, disks with wire diameter 1 mm and wire spacing of 3.15 mm; top picture, disk with 0.224 mm wires spaced 0.5 mm apart. Two spacers were put between tight meshes, one between wide meshes.

Results and Discussion

Shake Flask ISMS Experiments

Two shake flask cultures of *B. licheniformis* were conducted using complex medium. In both cultures the specific rate of protease production was low during the first 12 h, after which it accelerated (Fig. 5). The biomass concentration was 2.5 g L^{-1} (2.0 g L^{-1} in the reference shake flask) at this point of time. After 18 h significant amounts of proteases had been produced and the ISMS procedure was carried out using the culture from one of the two shake flasks as depicted in Figure 2. The results in Figure 5 show that the specific protease concentration was reduced by 64% from approximately 0.02 to 0.007 g protease g cell DW^{-1}. The reduction in protease concentration corresponded to a specific protein loading of 0.033 g g^{-1} on the adsorbents. A repeat experiment was conducted and similar results were found (data not shown).

Following ISMS, the culture continued to produce proteases at a similar rate to that before the separation procedure, and at a much higher rate than in the control cultivation (Fig. 5). The results suggest that removal of the proteases stimulated the organism to continue to produce the enzyme intensely. The total protease production was increased by more than 60% in the cultivation which had undergone ISMS. However, the final specific protease yield ($Y_{p,x}$, i.e., gram protease per gram cells) increase for the whole shake flask fermentation was only 30%. This is because the biomass concentration was higher in the shake flask which had undergone ISMS (3.1 g/L compared to 2.4 g/L). This difference even increased after ISMS. When the specific

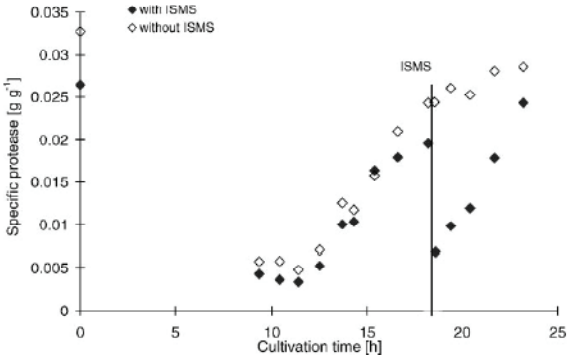

Figure 5. Specific protease production (g protease g DW^{-1}) in shake flask cultivation in complex medium with (◆) and without (◇) conducting ISMS after 18 h. The magnetic adsorbent concentration was 1.2 g L^{-1}.

production rates of protease (r_p) are compared before and after ISMS was conducted at 18 h, a large increase is seen: Before ISMS 2.1 (2.4) mg g DW^{-1} h^{-1}; after ISMS 4.7 (4.1) mg g DW^{-1} h^{-1} (values in brackets are from a repeat experiment). In contrast, the values for r_p decreased slightly for the reference shake flask before and after 18 h of fermentation: Before 18 h 2.7 (3.7) mg g DW^{-1} h^{-1}; after 18 h 1.8 (3.5) mg g DW^{-1} h^{-1}.

The above results demonstrate that ISMS can be applied during a fermentation without negative consequences for the culture. Indeed protease production and growth seem to be enhanced, which might be of considerable interest to industrial enzyme producers. Yet, the shake flask experimental conditions are not optimal compared to cultivation in a bioreactor. The pH value is not controlled, measurements are limited and sampling is disruptive (e.g., with respect to shaking and temperature control). Therefore, cultivations were carried out in bioreactors. The goals were primarily to examine more rigorously the effects of ISMS on the yield of protease formation given that the ISMS process seemed to enhance production.

Cultivation of *Bacillus licheniformis* in Bioreactors

Prior to studying ISMS, the characteristics of growth and protease production in the bioreactor were examined. The culture was allowed to grow as a batch cultivation for the first 23 h (see Fig. 6) after which the fed-batch phase was started. In the fed-batch a glucose-rich, ammonia poor medium was fed to the reactor ($q_f = 25.5$ mL h^{-1}) to ensure limitation of nitrogen, which is favorable for subtilisin induction (Pierce et al., 1992). The results showed that after a lag phase of circa 8 h *B. licheniformis* grew ($\mu_{max} = 0.36$ h^{-1}) to a concentration of approximately 15 g L^{-1} after 23 h and that little protease was produced (Fig. 6). In the following fed-batch phase little further growth was seen as nitrogen was limiting. However, protease production appeared to be induced and the specific protease content increased greatly in the extracellular medium. This procedure allowed higher amounts of proteases to be produced than in the shake flask cultures (0.05 g g DW^{-1} vs. 0.03 g g DW^{-1}) over a number of hours, whilst limiting increases in biomass. Thus it was concluded that a suitable fermentation strategy had been developed, which would allow the effects of ISMS on protease production to be examined.

ISMS During Bioreactor Cultivation

A nitrogen limited fed-batch cultivation was conducted in the same way as described above and the results in Figure 7 show that it proceeded in a similar fashion to that seen in Figure 6. Initially there was a lag phase of ca. 7 h (see Fig. 7) before the biomass concentration increased to ~9 g L^{-1} after 17 h. At this time the glucose became exhausted and protease production began. The feed (6 mL h^{-1}) was started 18.5 h after inoculation and the specific protease concentration in the bioreactor continued to increase linearly. Little further growth was seen due to the nitrogen limitation imposed. After 25.5 h the specific protease concentration was 0.03 g (g DW)$^{-1}$ and the ISMS procedure was carried out once. The procedure was analogous to that used with shake flasks. In this case, the total broth (ca. 2.25 L) was pumped into a new vessel, mixed with 4 g of adsorbents then pumped back to the fermenter via the magnetic separator (Fig. 3). The magnetic adsorbents were completely removed from the broth.

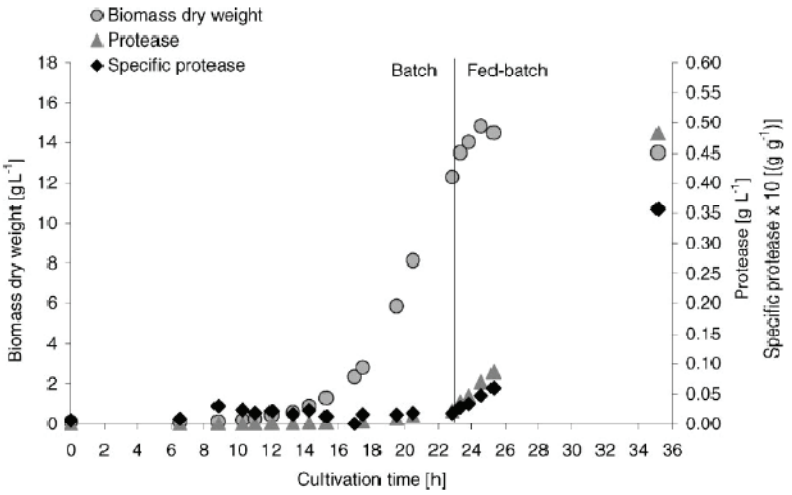

Figure 6. Fed-batch cultivation of *Bacillus licheniformis* in a bioreactor without ISMS. Synthetic medium was used and a constant feed of 25 mL h^{-1} was started after 23 h. Biomass dry weight (●), protease concentration (▲), specific protease concentration (◆).

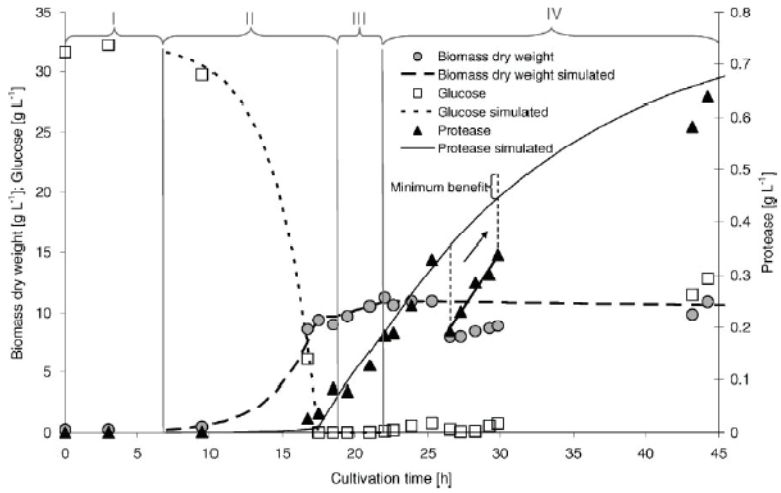

Figure 7. Fed batch cultivation of *B. licheniformis* in which ISMS was conducted after 25.5 h. Synthetic medium was used and a feed of 6 mL h^{-1} was started after 18.5 h. "Miniumum benefit" refers to the difference in total protease yield for cultures in which ISMS is conducted compared to those in which ISMS is not conducted; I, lag-phase; II, batch-phase; III, fed-batch with glucose limitation; IV, fed-batch with nitrogen limitation; dashed vertical lines: separated protease after ISMS in g L^{-1}.

It was found that after the ISMS procedure ca. 42% of the proteases had been removed, leaving 0.19 g L^{-1} in the broth. However since 27% of the biomass was also removed, this corresponded to a 19% drop in specific protease concentration (Fig. 7). Following the ISMS procedure, the specific protease concentration continued to increase at the same rate (0.04 g L^{-1} h^{-1}) as that observed before hand (i.e., 0.04 g L^{-1} h^{-1}). The oxygen limited conditions during ISMS therefore did not pose any drawback for protease production. Nevertheless, the biomass concentration was decreased after ISMS. Therefore specific protease production increased from 5 mg g^{-1} h^{-1} before ISMS to 7 mg g^{-1} h^{-1} after ISMS. Biomass loss can be traced back to two factors: The first is a dilution (<5%) which was caused by the sterile water used for washing the HGMS system after sterilizsation with 76% ethanol. In the boundary zone between the water and the culture broth some mixing took place. The second cause was that the HGMS system was very efficient at removing the magnetic adsorbents, which were primarily captured on the first meshes of the magnetic filter. The resultant "filter cake" of adsorbents entrained and also separated biomass from the suspension. The use of a magnetic filter in which the wires of the meshes (Fig. 4) were further apart would reduce this effect. Biomass might also have been adsorbed to the support surface.

The working capacity of the adsorbents (Q^*) was 0.066 ($\pm$0.011) g g^{-1}, which was much lower than expected from the adsorption isotherm (see Fig. 1 for $c^* = 0.19$ g L^{-1}), and also much lower than found in the ISMS experiment using shake flasks (0.033 g g^{-1} for $c^* = 0.022$ g L^{-1}). The reasons for this are unclear and could be due to a general reduction in the binding affinity in the fermentation broth compared to the single component system that the isotherm was determined in. The fermentation broth contains cells, other proteins and a variety of salts, sugars and medium components that can be expected to affect adsorption. Similarly, discrepancies between adsorption isotherms made in pure solutions and in real feedstocks have been previously reported (Franzreb et al., 2006). A second reason for low capacity could be due to non-specific adsorption of other proteins or even cells, thus restricting access of the protease to the ligands. It could be observed from SDS–PAGE gels (data not shown) that during the growth phase there was significant production of extracellular protein, in addition to the protease product. Furthermore, measurements of the total protein concentration in the fermentation broth (up to 0.2 g g DW^{-1}) showed there was much more protein than corresponded to the protease concentration. The overall protein selectivity of the adsorbent (S) was determined to be $S = 1.9$ in an additional experiment, where extracellular protease made up 14% of extracellular protein. The following relationship was used:

$$S = \frac{m_{\text{Protease,ads}} / m_{\text{Protease,total}}}{m_{\text{Protein,ads}} / m_{\text{Protein,total}}} \quad (1)$$

This selectivity is quite low for an affinity adsorption and seems to contradict the isotherm shown in Figure 1. One explanation could be non-specific binding of proteins to the adsorbent, which thereby reduces protease binding. Sites for non-specific adsorption could have arisen from the adsorbent preparation method used. Glutaraldehyde was used to coat the support prior to activation with DVS. It is unlikely that all of the available coupling sites reacted with the small peptide ligand used and these may thus be available

for non-specific binding interactions. Multilayer protein binding to adsorbed proteases or other proteins could also distort the binding properties of the adsorbents. Some evidence for non-specific adsorption was seen when the washing fractions were analysed following adsorbent capture. The results suggest that improvements in adsorbent construction and ligand selection are required in further work. However, a rigorous examination of the surface properties of the adsorbents constructed here was not the focus of the current work. Rather, it was the successful demonstration of the ISMS concept.

Protease Recovery During Elution

Following the capture of the adsorbents in the magnetic filter, they were washed once and the proteases eluted in two steps using the setup described earlier in Figure 3. These procedures took place in the HGMS closed loop which had a total working volume of 0.68 L including the filter (Fig. 3). In this case the adsorbent mass to elution volume ratio was 5.9 g L^{-1} and each pool from the wash or elution was 0.68 L. Washing was conducted for 1 h and resulted in the loss of only 6% (i.e., 0.016 g) of the proteases. During each of the two consecutive elution steps the particle suspension was continuously pumped through the closed loop at a rate of 1 L min^{-1} for 1 h. In this case 50% of the adsorbed protease could be desorbed in an active state after two elution steps. At the end of the complete procedure, 94% of the adsorbents were recovered: 4% were lost during washing and 2% were lost during elution.

During the HGMS procedure, only 50% of the captured proteases were eluted, thus further studies were conducted to determine the reasons for this. Other workers have shown that poor elution efficiencies from HGMS filters can be due to a number of factors, such as poor particle release from the filter, lack of mixing in the recycle loop, unfavorable equilibrium and deactivation of the product. The batch elution experiments conducted here were designed such that all of these potential problems could be reduced. Additional experiments were performed in small scale in 1.5 mL reaction vessels: In this case magnetic adsorbents of the same kind as used above were loaded with protease from cultivation broth, washed and then eluted. During each elution step the reaction vessels were mixed vigorously on an orbital shaker at 300 rpm for 30 min at 22 or 37°C. The particle-mass to elution-volume ratio was reduced from 5.9 g L^{-1} (in the HGMS procedure) to 1 g L^{-1} (in the small scale) to improve the desorption equilibrium. By making these simple changes, it was observed that after the first and second elution steps 81(±6)% and 94(±6)% respectively, of the bound protease was desorbed. There was no notable difference in desorption behavior when 22 or 37°C was used. The improvement in elution efficiency is believed to be due mainly to better mixing in the shaker, although the lower particle-mass to elution-volume ratio in the small reaction vessels can also improve the desorption equilibrium. However, this leads to considerable dilution of the product, which is not desirable. Thus, in the following section, the assumption will be made that all of the protease captured using ISMS could be completely recovered in an active state in the subsequent elution steps. Therefore the total protease yield of the bioproduction should be calculated by adding the amount of protease removed by ISMS to the amount of protease in the reactor at the end of the cultivation. Comparison with a fermentation in which ISMS was not used would then permit the benefits of ISMS on total protease yield for the cultivation to be shown. However, it is not a trivial matter to predict what the yield of protease would have been for the fermentation shown in Figure 7 if ISMS had not been used. To make this prediction, a model is needed to simulate the fermentation performance in the absence of ISMS to obtain the data needed.

Fermentation Simulation and Benefit of ISMS for Protease Production

Protein Production Kinetics

The production of proteins in a fermentation is often described by using an approach from Luedeking and Piret who include a growth dependent (β) and a growth independent (α) term, representing the true yield coefficient (Y_{PX}^{true}) and the specific rate of protein production due to maintenance, respectively (Luedeking and Piret, 1959):

$$r_p = \alpha + \beta\mu \tag{2}$$

The specific production rate r_p can then be inserted into the differential equation which is obtained from a mass balance considering the produced protein and which is valid for batch ($D = 0$) or fed-batch operations ($D > 0$):

$$\frac{dc_p}{dt} = r_p c_x - D c_p - k_{deg} c_p \tag{3}$$

Here c_p and c_x are the concentrations of protein and biomass, respectively. D is the dilution rate and k_{deg} is the coefficient of protein degradation. The first term on the right hand side of the equation is the productivity of protein by the organisms, the second term considers dilution resulting from substrate addition during fed-batch operation and the third term incorporates degradation of the protease due to instability or auto-proteolysis (Kaufmann, 1997; Perler et al., 1997). Equation (3) does not include a product inhibition term. It could, however, be relevant in systems where accumulating products affect the metabolic pathways and reduce growth or production rates. For example, for proteases it has been reported that product accumulation causes end-product repression, which is not caused by direct interactions between the cell and protease. Rather it results from hydrolyzed products in the medium such as the cleaved amino acids and peptides (Priest, 1977). Thus,

removing proteases from the cultivation would not only lead to lower proteolytic activity, hence stabilizing other proteins, but would also reduce formation of amino acids in the broth, which might otherwise inhibit production (Calik et al., 2003).

For simulation of the fed-batch fermentation shown in Figure 7 the uptake of each substrate was linked to the formation of biomass and was based on the unstructured Monod model (Eqs. 4–7). Product formation was simulated by implementing Equations (2) and (3) into the model. To simplify the simulation, the cultivation shown in Figure 7 was divided into four parts: phase I and II describe the lag and batch phase, respectively, which were followed by two fed-batches, phases III and IV corresponding to the periods of glucose limitation and nitrogen limitation, respectively (Fig. 7). The lag phase (phase I) was not simulated. The course of glucose was simulated for phase II and III whereas the course of biomass and protease were simulated for phases II, III, and IV. The calculation was carried out with Matlab (Mathworks, Natick, MA).

$$r_S = r_{S,max}\frac{c_S}{c_S + K_S} \tag{4}$$

$$\mu = Y_{X,S} r_S \tag{5}$$

$$\frac{dc_S}{dt} = -r_S c_x + Dc_{S,f} - Dc_S \tag{6}$$

$$\frac{dc_X}{dt} = \mu c_x - Dc_X \tag{7}$$

For the simulation, the parameters used in Equations (2)–(7) are required. For use of Equation (2) the parameters α and β are needed. These were determined from plots of r_p versus μ (excluding degradation) for several fed-batch fermentations. Since the productivity was increasing with decreasing specific growth, due to the nitrogen or glucose limitation, the growth specific parameter β was negative. Equation (3) defines the general mass balance for protease and includes a product degradation term. Product instability was thus investigated and it was found that the protease was subject to first order degradation kinetics both in buffer and in medium supernatant from the bioreactor. The degradation constant could be determined in fermentation broth and buffer as $k_{deg} = 0.064\ h^{-1}$.

Here yield of biomass as a function of substrate consumed ($Y_{X,S}$) was calculated from batch phase data from several cultivations for the substrates glucose and ammonium. The specific growth rate μ was calculated for both substrates and the smaller value was used if there was a difference. In this way, the effect of growth due to the substrate that was limiting was accounted for. During phase III the biomass increased from 9 to 11 $g\,L^{-1}$ due to glucose uptake (Fig. 7). After 22 h of cultivation glucose was no longer the limiting substrate. Instead a nitrogen limitation prevented considerable growth of biomass (concentrations of NH_4^+ not shown in Fig. 7). The system of Equations (2)–(7) has a limitation, for example, for $c_S = 0$. Under this condition, the model predicts product formation without any substrate uptake. However, the equations are often used in literature (e.g., Millitzer et al., 2005). In phases when glucose substrate concentration is very low there can be the transition of storage substances or substances produced in the batch phase to the product. So stoichiometry is not violated.

The parameters used for the simulation are given in Table I and the results (Fig. 7) show that the simple model used here gives a very good approximation of the experimentally determined time courses for c_P, c_x, and c_G. It was therefore deemed appropriate to use the simulation to predict the production of protease in Figure 7, if ISMS had not been conducted. The concentration predicted was 0.45 $g\,L^{-1}$ at 30 h (Fig. 7). In contrast, the total yield of proteases actually observed as a consequence of using the ISMS procedure was 10% higher. This value was determined by adding the drop of protease concentration after ISMS (0.164 $g\,L^{-1}$; Fig. 7, vertical dashed line at 26.5 h) to the amount which was present in the reactor (0.338 $g\,L^{-1}$) (Fig. 7, arrow and vertical dashed line at 30 h). This latter value assumes that the protease production rate during the ISMS procedure was not diminished, which is a reasonable assumption given that the rate after ISMS is the same as that before. The yield increase shown in Figure 7 due to ISMS would be expected to be considerably higher if biomass loss could be avoided. Thus despite the loss of some biomass, considerable advantages with respect to protease yield are seen by using ISMS. Further work should be aimed at improving protease capture whilst minimizing biomass removal.

Perspectives of ISMS for Commercial Use

Use of one cycle of the ISMS procedure was shown to be effective for not only removing the protease product, but also for increasing the fermentation yield. This is likely to be of great interest for the production of low value bulk enzymes such as those produced using *Bacillus*. It can be expected that further ISMS process optimization and use of repeated or continuous ISMS steps may provide even greater benefits and this should be studied in further work. The

Table I. Parameters used for bioprocess simulation shown in Figure 7.

Parameter	Determination method	Value
μ_{max}	Measured	0.36 h^{-1}
$Y_{X,G}$[a]	Measured	0.3 $g\,g^{-1}$
$Y_{X,N}$[a]	Measured	3.55 $g\,g^{-1}$
$K_{S,G}$, $K_{S,N}$	Estimated	0.1 $g\,L^{-1}$
k_{deg}	Measured	0.064 h^{-1}
α	Measured	0.005 $g\,g^{-1}\,h^{-1}$
β	Measured	−0.013 $g\,g^{-1}$
q_F	Set	6 $mL\,h^{-1}$

[a]Biomass formed per gram of glucose or ammonium, as appropriate.

reasons for the increased yield of protease are believed to be due to the ability to avoid autoproteolytic degradation of the protease during the fermentation. However it can also be envisaged that similar benefits of ISMS may be expected if the substance to be removed inhibits the fermentation. Strains from the genus *Bacillus* naturally have a very higher secretion capacity and could potentially be used for heterologous protein production. However, one major obstacle when using these host systems for heterologus protein synthesis is their natural protease production. The proteases are present in cultivation broths of *Bacillus* both in the medium and on the cell wall (Li et al., 2004; Vitikainen et al., 2005; Westers et al., 2004). The destructive effect of these enzymes during heterologous protein production could potentially be diminished by ISMS.

Application of ISMS in an industrial setting requires that several challenges be overcome. In particular, energy consumption during magnetic separation, particle properties such as specificity, sterilisability and reusability, as well as process optimization such as the conditions used for adsorption and elution and particle separation. There are however, approaches to manage these challenges. Energy consumption could be reduced dramatically by using switchable permanent magnet systems as was described previously (Hoffmann et al., 2002) or by using superconducting magnets. Furthermore, new separator types such as a magnetically enhanced centrifuge have recently been reported (Stolarski et al., 2007) and promise to considerably increase throughput for industrial use. Improvements in the properties of magnetic particles are being studied in many research groups (Banert and Peuker, 2007; Ditsch et al., 2006; Lattuada and Hatton, 2007). Work is also required to develop suitable affinity ligands, which are stable, functional in the conditions present in the fermentation broth and which selectively remove the one target component of interest. This high affinity must, however, still allow efficient elution to recover the product.

Conclusion

The ISMS process is a new promising ISPR method for removing substances during a cultivation. It could be shown that proteases could be recovered from shake flask cultures using the method. This separation was beneficial for the overall yield of the enzyme. ISMS was also successfully scaled-up by using HGMS and integrating it with a bioreactor. The magnetic adsorbents used for protease capture could be completely removed from the biosuspension and recovered after subsequent processing operations including washing and elution steps. The degrading properties of the protease in the fermenter could be reduced using ISMS and it was shown that removing the proteases resulted in an overall enhancement of protease production and recovery. For industrial use ISMS needs significant further development. Nevertheless, the ISMS procedure shows great promise for intensifying bioproduction when the extracellular products produced are subject to degradation in the broth, negatively interfere with the microbial cells or with other extracellular components.

Nomenclature

c	concentration ($g\,L^{-1}$ or $mol\,L^{-1}$)
D	dilution rate (h^{-1})
F	force (N)
H	magnetic field ($A\,m^{-1}$)
k_{deg}	degradation coefficient (s^{-1})
K_d	dissociation constant ($g\,L^{-1}$ or $mol\,L^{-1}$)
K_S	Monod constant ($g\,L^{-1}$)
m	mass (g)
M	magnetization ($A\,m^{-1}$)
Q	support capacity ($g\,g^{-1}$)
q	volume flow ($mL\,h^{-1}$)
r	specific production uptake rate ($g\,g\,DW^{-1}\,s^{-1}$)
S	selectivity
t	time (s)
T	temperature (K)
V	volume (mL)
Y	yield coefficient ($g\,g^{-1}$)

Greek Letters

α	growth-independent production coefficient ($g\,g^{-1}\,s^{-1}$)
β	growth-dependent production coefficient ($g\,g^{-1}$)
μ	specific growth rate (h^{-1})
μ_0	permeability of vacuum ($4\pi \times 10^{-7}\ V\,s\,A^{-1}\,m^{-1}$)

Indices

*	equilibrium state
ads	adsorbed
f	feed
G	glucose
max	maximum
N	ammonium
P	protease or Protein
S	substrate
x	biomass

Abbreviations

BSA	bovine serum albumin
DAAD	German Academic Exchange Service
DSMZ	German Resource Centre for Biological Material
DVS	divinylsulfone
DW	cell biomass dry weight
HGMS	high-gradient magnetic separation
ISMS	in situ magnetic separation
ISPR	in situ product removal
MP	magnetic particles
OD	optical density
RPM	rotations per minute

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